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Development of rhizoremediation as a treatment technology in the removal of Polycyclic aromatic Hydrocarbons (PAHs) from the environment

By

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I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirely or part has been submitted at any university for a degree

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List of Abbreviations

P – Phosphorus

Mn – Manganese

Zn – Zinc

Cu – Copper

PAHs – Polycyclic Aromatic Hydrocarbons

UV- Ultra- Violet

PCP – Pentachlorophenol

BaP – Benzo(a)Pyrene

PCBs – PolyChlorinated Biphenyls

TSB – Tryptone Soy Broth

TSA – Tryptone Soy A gar

DNA – Deoxyribonucleotide acid

O_2 – Oxygen

Arg – Arginine

Orn – Ornithine

HC – Hydrocarbon

GC-MS – Gas Chromatography- Mass Spectrophotometer

HPLC – High Performance Liquid Chromatography

rRNA – ribosomalRibonucleic acid

CLPP – Community Level Physiological Profile

LMW – Low Molecular Weight

HMW – High Molecular Weight

N_2 – Nitrogen

C- Carbon

S – Sulphur

diCBA – diChlorobenzoic acid

FAME – Fatty Acid Methyl Ester

G+C – Guanine+ Cytocine

PCR – Polymerase Chain Reaction

RAPD – Random Amplified Polymorphism DNA

DGGE – Denaturant Gradient Gel Elecrophoresis

VOC –Volatile Organic Compound

PLFA – Phospholipids Fatty Acid

T –RFLP – Terminal –Restriction Fragment Length Polymorphism

ITS –Intergenic Transcribed Spacer

ppm – part per million

AWCD – Avarage Well Colour Development

MVA – Multi Vatiate Analysis

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Chapter 1

Introduction

1.1 The role of microbial communities in soil

Soil is one of our most valuable resources. It regulates global biogeochemical cycles, filters and remediates pollutants and enables food production. One particular significant component of soil is microorganisms (Borneman *et al*., 1996). Kennedy *et al*., 1995, described some of the key processes that are controlled by these microorganisms including the decomposition of organic material and plant residues, increased nutrient availability of P, Mn, Fe, Zn and Cu, nitrogen fixation, biological control of pests, biodegradation of pesticides and pollutants and finally improved soil aggregation.

Microorganisms are vital to the function and maintenance of the ecosystem and biosphere. Microorganisms can be used for the biomonitoring of the global ecosystem, cleaning of environments (bioremediation), for climatic changes, for the effects of pollutants and other habitual disturbances (Atlas, 1984).

Microbial communities in soil are made up of five major groups, namely: bacteria, archaea, fungi, algae and protozoa. Bacteria are the most abundant group and usually more numerous than the other groups. The number of bacterial cells in soil is always high. In adequate aerated soil the bacteria and the fungi dominate whereas bacteria alone account for almost all the biological and chemical changes in environments containing little or no oxygen (Jensen *et al*., 1986).

Soils are reported to contain 10^5- 10^8 bacteria, 10^6- 10^7 actinomycetes and 10^5- 10^6 fungal colony forming units g^{-1}. These high numbers together with the limitations of conventional plating techniques pose a great problem in

studying microbial populations in soils. Bacteria that are able to grow on agar plates typically account for only around 1% of the bacteria in the soil. Also, the presence of an organism in the environment does not necessarily mean that it is contributing significantly to soil processes (Lawlor *et al*., 2000).

Bacteria in soil can be placed into two divisions: the autochthonous or indigenous species and the zymogenous or fermentative organisms. The autochthonous population consists of the numerous indigenous bacteria whose abundance is not subject to environmental fluctuations. Environmental conditions affect the density and composition of the bacterial flora, and non- biological factors can alter to a great degree the nature of the population and its biochemical potential. The primary environmental variables influencing soil bacteria include moisture, aeration, temperature, organic matter, acidity and inorganic nutrient supply (Atlas *et al*., 1984).

Zymogenous organisms are those that are most active in chemical transformations and are usually scarce but they flourish when organic nutrients are added. Their nutrient supply is derived from the soil organic fraction and no external nutrients or energy sources are required, hence their numbers remain relatively constant. The actively fermenting species therefore need nutrients provided for their growth. But these organisms will decline once their food sources are depleted (Insam *et al*., 1997).

Some of the bacteria persist in unfavourable conditions by the formation of endospores that function as part of the normal life cycle of the bacterium. These endospores often endure in adverse environments because of their great resistance to both prolonged desiccation and to high temperatures. The endospores can persist in a dormant state long after the

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lack of food or water has led to the death of vegetative cells. When conditions conducive to vegetative growth return, the spore germinates and a new organism emerges (Atlas *et al*., 1984).

1.2. Microbial diversity in soil

The need exists because of the lack of knowledge about the diversity and function of microbial communities due to the fact that a need exist for effective methods to evaluate microbial community structure. Isolate-based techniques have often been used, but they offer a limited, biased view of microbial communities (Gerland, 1997).

Molecular and biochemical techniques enable identification and phylogenetic characterization of microorganisms without cultivation. Other approaches for fingerprinting microbial communities are DNA-based method such as Denaturant Gradient Gel Electrophoresis (DGGE), Terminal Restriction Fragment Length Polymorphism (T-RFLP) and Intergenic Transcribed Spacer (ITS). However, these methods are time consuming, complex and it is difficult to interpret the data obtained from these methods regarding diversity of microbial communities (Ellis *et al*., 2001).

Biolog System has been used extensively as a simple and rapid community level tool to characterize and classify microbial communities based on the sole carbon source utilization patterns. Simplicity of this method and the commercial availability of the microtiter plates make this approach very attractive (Gerland, 1997).

The determination of bacterial species diversity is an important aspect of

environmental studies. Diversity decreases in communities under stress (Atlas, 1984). Microbial diversity describes complexity and variability at different levels of biological organization. It encompasses genetic variability within taxons (species), and the number (richness) and relative abundance (evenness) of taxons and functional groups in communities. Important aspects of diversity at the ecosystem level are the range of processes, complexity of interactions and number of trophic levels. Diversity may also be considered to be the amount of distribution of information, which is directly applicable to the total genetic diversity or complexity in a community (Stephan *et al*., 2000).

Evaluation of diversity in environmental microbial communities has been limited by the large percentage of microorganisms that are non-culturable. Knowledge of the species composition of microbial communities can provide insight into the ecological function of these communities and assist the choice of suitable methods for remediation of polluted system (Insam *et al*., 1997).

Gerland *et al*. (1991) introduced the Biolog system (Biolog Inc., CA, USA) as a rapid community level tool to characterize and classify heterotrophic microbial communities based on the carbon source utilization pattern. Microtiter plates are commercially available that contain a large number of metabolic tests. The biolog system is based on 95 individual carbon source oxidation tests, for which the colour change of the redox dye tetrazolium violet is used as an indicator of utilization of the carbon source (Gerland, 1997). Functional diversity is measured with a substrate utilization assay such as the Biolog system and can be determined in terms of rates of utilization, the presence or absence of utilization and can be determined for any number of potential substrates (Cloete *et al*., 1998).

Simplicity of the method and the commercial availability of the microtiter plates make this approach very attractive. Consequently, the Biolog System has been used to characterize microbial communities of soil, water, plant rhizosphere and phyllosphrere microbial communities, activated sludge and composted manure (Verschuere *et al*., 1997, Cloete *et al*., 1998).

1.3. PAH/ PCB pollution and effects

Polycyclic aromatic hydrocarbons (PAH_S) are pollutants of great environmental concern
because of their toxic, mutagenic and carcinogenic properties. The Biodegradation of PAH_S, particulally those of low molecular weight, has been widely investigated. It has been shown that PAH_S can be totally degraded or particularly transformed either by a single bacterium or community of bacterial species (Cuypers *et al*., 2002).

Polycyclic aromatic hydrocarbons (PAH_S) adsorption onto organic particles of sediments can cause carcinogenic PAH_S to reach much higher concentrations in the sediment than in the upper water column. PAH_S are introduced to animals and humans from the food chain through the digestion system (Rababah *et al*., 2002). Clarke *et al*. (2000) determined

high risk posed by consumption of seafood marine prey species contaminated with fluoranthene to humans and to Chinese white dolphin (*Sousa chinenses*). Cole *et al*. (2000) reported that the toxicity of PAH_S (dry weight) in sediments to marine *Rhepoxynius abronium* was enhanced by UV irradiation during 27- 83 days of exposure.

Benzo(a)pyrene (BaP), naphthalene, phenanthrene and related polycyclic aromatic hydrocarbons (PAH_S) are ambiquous environmental contaminants. PCP, a persistent compound introduced into the environment through its use as a disinfectant, herbicide, insecticide and fungicide has been linked to reproductive abnormalities and thyroid dysfunction. PAH_S commonly enter the environment as incomplete combustion byproducts of carbonaceous materials and may persist in groundwater, surface water, soil, activated slugde, air and foodstuffs (Ake *et al*., 2003).

Chlorinated organic chemicals probably constitute half of the environmental organic pollutant problems in the world (Tiedje *et al*., 1993). Over the past 20 years, numerous studies have reported the capacity of various bacteria, fungi and algae to degrade polycyclic aromatic hydrocarbons (PAH_S) and polychlorinated biphenyls (PCB_S). However, few studies have focused on the contribution of individual microorganisms in reducing the toxicity of PCB/PAH compounds. Microbial degradation of PAHS may result in the incomplete breakdown of the compound depending on the environmental conditions and the microbial population present (Juhasz *et al*., 2000).

Different degradation techniques may be used for the remediation of PAH-contaminated matrices. A suitable degradation technique however, depends on many factors, such as required duration of treatment, cost, type of environmental matrix, site sensitivity, climate, PAH molecular

weight and concentration (Rababah *et al*., 2002).

The most common degradation techniques that have been widely investigated are hypothermal, biodegradation, phytoremediation and photodegradation. This technique however, may be limited by the high cost of maintaining supercritical conditions and reactor's material that must withstand the high pressure and temperature (Shanableh *et al*., 2000).

Nonetheless, microorganisms may be at risk from the high levels of PAHs in soils and sediments under certain environmental conditions (e.g. UV exposure). Moreover, PAH_S are quite resistant to degradation. Their half-lives in soils may reach 28 years depending on the compound. The fact that fluoranthene is a high molecular weight PAH that is dominant and persistent in sediments and soils, indicates that biodegradation may not be an ideal treatment option in some cases (Rababah *et al*., 2002).

1.4. Rhizoremediation

Remediation of soil contaminated with organic chemicals using techniques such as incineration and landfilling can be expensive. An alternative technique which involves the mutualistic relationship between plants and microorganisms to breakdown contaminants was employed to clean- up the environment. This technique is called rhizoremediation (Nichols *et al*., 1996).

Rhizoremediation is a technique that is used to clean up the environment contaminated with hazardous pollutants. Plants remediate organic pollutants by: 1) direct up- take of contaminants, which are converted into non- toxic substances and accumulated in the cells 2) release of exudates and enzyme stimulating microbial activity, growth and biochemical transformation 3) enhancement of mineralization in the rhizosphere

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(Mackova *et al*., 1997).

Rhizoremediation is an effective, non-destructive and inexpensive means of remediating environment (Chaney *et al*., 1997). It is more cost effective than other methods such as mechanical and chemical methods of removing hazardous compounds from the environments (Pradhan *et al*., 1998). Besides, rhizoremediation is a natural, aesthetically pleasing low cost technology. It is socially accepted by surrounding communities and regulatory agencies as a potential elegant and beautiful technology (Alkorta *et al*., 2001).

Work of Liste and Alexander (2002) have shown that plants promoted the degradation of 74% of pyrene from vegetated soil within eight weeks compared to less than 40% degradation from non-vegetated soils.

Biodegradation is effective under aerobic conditions. Aerobic biodegradation is more favourable, because it is faster compared to anaerobic biodegradation (Rockne *et al*., 1998). Joshi and Lee (1996) have shown that 80% of PAH from contaminated soil was aerobically degraded by microorganisms after seven days in controlled laboratory conditions.

Plant rhizospheres are ideal locations for studies of competitive interactions of microorganisms. Rhizospheres are dynamic microenvironments in which microbial communities have access to an elevated supply of carbon and energy rich materials from the plant roots to the bulk soils and sediments (Clegg *et al*., 2002).

These communities supported by high levels of carbon resources should be capable of both quantitative and qualitative changes in composition. Rhizospheres are also stable physically, avoiding the potentially compounding effects on naturally occurring disturbances on microbial community composition or activities (Piceno *et al*., 2000).

Studies based on the hypothesis that root exudates increase the rhizosphere microbial community was made, that investigated the significance of plant microbial interactions for the degradation of PAH_S. Walton *et al*. (1994) speculated that when a chemical stress is present in the soil, a plant may respond by increasing or changing exudation to the rhizosphere, which modifies rhizosphere microflora composition or activity. As a result, the microbial community might increase the transformation rates of the toxicant.

Gunther *et al*. (1996) noted that in a soil polluted with PAH_S and aliphatic hydrocarbons, microbial plate counts and soil respiration rates were higher in the rhizosphere of ryegrass than in the bulk soil. Reilley *et al*. (1996) showed that degradation of pyrene increased in the rhizosphere soil and that the highest pyrene mineralization rate was found when organic acids, typically found in the root exudates, were added to the soil.

Nichols *et al.* (1997) showed that some bacteria grew and multiplied in a selective enrichment medium amended with a mixture of PAHs and had the ability to degrade PAH_S in the rhizosphere of *alfalfa* and bluegrass.

However, Chaineau *et al*. (1995) showed a rapid adaptation of the soil microbial community to degradation of hydrocarbons in an agricultural field plot amended with drill cutting, and a specific diversity of the degraders, but did not compare rhizospheric and non- rhizospheric soil.

1.5. Phytoremediation

The enormous growth of industrialization and the use of numerous aromatic compounds in dyestuff, explosive and pharmaceuticals have resulted in serious environmental pollution and have attracted considerable attention continuously over the last decade. Many aromatic hydrocarbons, nitroaromatic compounds, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, diauxins and their derivatives are highly toxic, mutagenic and / or carcinogenic to natural microflora as well as to the higher systems including humans (Singh *et al*., 2003).

The increasing costs and limited efficiency of traditional physicochemical treatments of soil have spurred the development of new remediation technologies (Huang *et al*., 2004). Therefore, an alternative technology that is low cost, self sustain remediation, solar driven and environmentally friendly is needed. Such technology is called phytoremediation. Phytoremediation may be defined as the use of plants to remove or destroy hazardous substances from the environment (Pulford *et al*., 2003).

Phytoremediation is applicable for treating a wide variety of contaminants and is well suited for sites with shallow, contaminated soil, streams and ground water (Singh *et al*., 2003).

A number of different types of plants are effective at stimulating the

degradation of organic molecules in the rhizosphere. Typically, plants have extensive and fibrous roots, which form an extended rhizosphere. Plants include common grasses as well as corn, wheat soybean, peas and beans. In addition, several varieties of trees can take up and degrade some organic contaminants. For example, plants with phytotransformation activity may contain nitroreductases, which are useful for degrading TNT and other nitroaromatics, dehalogenases for the degradation of chlorinated solvents and pesticides and laccases that can degrade anilines such as triaminotoulene (Glick, 2003).

A number of plants that can naturally accumulate large amount of metal have been identified in the study of phytoremediation. Those plants are called hyperaccumulators and are often found growing in areas with elevated metal concentrations in the soil. In the presence of very high concentrations, hyperaccumulating plants attain only a small size. That is, high concentration of metal concentrations are inhibitory to the growth of plants even those plants that are capable of hyper accumulating metals (Glick, 2003; Pulford *et al.*, 2003).

Phytoremediation of metals and other inorganic compounds may take one of several forms: phytoextraction, the adsorption and concentration of metals from the soil into the roots and shoots of the plants, rhizofiltration, the use of plant roots to remove metal from effluents, phytostabilization, the use of plants to reduce the spread of metal in the environment or phytovolatilization, the uptake and release to the atmosphere of volatile materials such as mecury or arsenic containing compounds (Huang *et al.*,

2004).

Phytoremediation of organic compounds may occur by phytostabilization, phytostimulation, the stimulation of microbial degradation in the rhizosphere, the area around the roots of plants, or by phytotransformation of contaminants by plants (Glick, 2003).

1.5.1. Advantages of phytoremediation

A primary advantage of phytoremediation is that the use of vegetation is usually well received by the public. Communities would rather support a mass planting of plants and tress than a landfillor an excavation process (Matso, 1995). Furthermore, phytoremediation generates less secondary waste and fewer air emissions. In addition, the soil and ground water remain in place and are reusable following treatment (Chappell, 1996).

1.5.2. Disadvantages of phytoremediation

Like any other technology, phytoremediation has some disadvantages. The primary disadvantages include, the time required to achieve clean up, the limited depth of treatment, toxic effect on plants, the transfer of contamination across media and regulatory acceptance. With phytoremediation, the time required for remediation can be a problem, because the process may require a longer period than other remedial approaches to achieve acceptable clean up levels. In addition, phytoremediation is limited to the depth that the plant roots can reach and to sites with low contaminants concentrations because concentrations that are to high can be toxic to plants. Phytoremediation can also cause an undesirable transfer of the contaminant from one type of media to another, such as transfer from water to the air during evapotranspiration. Finally, because phytoremediation is a new technology, regulators are

unfamiliar with it and may impose restrictions (Federal Remediation Technology Roundtable, 1998; http://lamar.colostate.edu/~samcox/phytoremediation.html).

Phytoremediation has received considerable attention because of it offers a cheaper, easier and environmentally sound pollution remediation option. Seeding a field of plants and harvesting them to extract the pollutants is much cheaper than removing huge amounts of contaminated soil. Phytoremediation is non- invasive and does not require heavy machinery to excavate soil. For this reason, it is aesthetically more pleasing. Excavating and transferring contaminated soil or water to a hazardous waste storage facility is simply moving problem without cleaning anything, whereas phytoremediation is real remediation of the substrate (http://lamar.colostate.edu/~samcox/advantages.html).

The aim of this study was to employ the Biolog system to determine microbial diversity of the rhizosphere of selected plants used in a rhizoremediation study were PAH/PCB contaminated and uncontaminated soils were sampled to test the hypothesis that microbial diversity is reduced under environmental stress, and that selection of tolerant species takes place, and could possibly be responsible for biodegradation of the pollutants.

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Chapter 2

Literature review

2.1. Soil microbial communities

Microorganisms mediate soil processes important to soil quality, such as regulating organic matter decomposition and nutrient availability, initiating and maintaining soil structure and removing organic contaminants from soil. Microbial processes such as soil respiration, metabolic profile and enzyme activities are commonly considered in the study of microbial communities because they rapidly respond to change in the soil environment. Information about the composition and diversity of microbial communities can also be used in the study of microbial communities (Johnson *et al*., 2003).

The biodiversity of the soil microbial community is important to maintenance of soil ecosystem function. The very large number of microbial species present in soil and difficulties with their culture and with species differentiation has led to development of approaches based on the biodiversity of communities rather that species. One such technique involved analysis of community functioning based on utilization carbon substrates (Nsabimana *et al*., 2004).

In recent times, attention has focused on the impacts that agriculture land use has on biological and biochemical properties of soil. Soil is a dynamic living resource and biologically mediated processes are central to its

ecological function. Key soil microbial processes include degradation of organic residues, transformations of soil organic matter, mineralization and immobilization of nutrient and formation and stabilization of soil aggregates (Aimas *et al*., 2004).

Organic and inorganic fertilizer amendments are used primarily to increase nutrient availability to plants, but they can also affect soil microorganisms. Organic matter addition often leads to a rapid increase of soil CO2 evolution and increase the activity of various enzymes. An increase in enzyme activity was also observed after addition of farmyard manure in a long- term study. Amendments can change amount and quality of dissolved organic matter present in the soil. Dissolved organic matter is an easily available substrate for soil microorganisms (Aimas *et al*., 2004).

Amendments usually increase the amount of soil organic compound carbon and concentration of other nutrients such as nitrogen. Microbial biomass increases with increasing organic carbon content of the soil. Different types of amendments may differ in organic matter composition (e.g. C/N ratio), and this in turn affects the decomposition rate and can change the microbial community structure. The increase in catabolic diversity that is observed in soil after addition of degradable organic matter also indicated change in microbial community structure (Marschner *et al*., 2003).

Trace metal contamination may result in an increased uptake by plants and affect the functions and composition of soil microbial communities. Microbial functions in soil are quite resilient towards metal contamination i.e. they are often found to recover after an initial inhibition by high metal concentrations. Two factors may contribute to these recoveries. One is a gradual decrease in metal availability due to immobilization reaction in soil. But more important metal tolerant organisms may replace the metal sensitive once within each functional group, thus changing the microbial community's composition and increasing its metal tolerance (Marschner *et al*., 2003).

Such changes in microbial structure and the tolerance of metals appear to be very sensitive indicators of the biological availability of metal in soil. Studies of metal contaminations was made and reported the gradual change in microbial community structure, based on changes in phospholipids fatty acid profiles, as a effect of trace metal contamination. These changes were detected at metal concentrations much below those affecting ATP and respiration (Aimas *et al*., 2004).

The ratio of microbial biomass carbon to total organic carbon has been used as an indicator of changes in organic matter status that will occur in response to alterations in land use, cropping system, tillage practice and soil pollution. Indices of microbial activity include basal respiration rate (CO2 evolution), general indicators of enzymatic activity such as arginine ammonification rate and fluorescene diacetate (FDA) hydrolysis, the activity of endocellular enzymes such as dehydrogenase and the activity of specific exocellular enzymes involved in nutrient transformations (e.g. acid phosphatase and anylsulphatase). The microbial biomass is the living component of soil organic matter and it typically comprises 1- 5% of total organic matter content. Because of its high turnover rate, microbial biomass carbon content can respond rapidly to change in soil management practice (Aimas *et al*., 2003).

Comparative studies have documented that microbial communities can change in respond to soil disturbance and differences have been observed between microbial communities in fields with different histories of soil amendment, irrigation, tillage and plant community structure (Buckley *et al*., 2001).

2.1.1. Function of individual species within the community

The concept of 'Keystone' species implies that there are some species or a group of species that perform important ecosystem functions, e.g. nitrifiers. However, it is not simply a question of whether these species are present. The activity of these individual species is affected by the composition of the microbial community in which they reside (Atlas, 1984).

Soil microbial communities vary depending on soil physical and chemical properties, type and amount of plant cover and climate. It is well known that plants influence the biodiversity of bacteria in soils. Through the release of compounds such as amino acids, sugars and growth factors in plant root exudates, microbial activity and growth are stimulated. Because bacteria respond differently to these compounds, differences in the composition of root exudates can influence the type of bacteria present in the rhizosphere community. Although, all plants show a rhizosphere effect, the plant species can influence the types of bacteria that are present in the rhizosphere (Dunfield *et al*., 2001).

2.1.2. Interaction among species within the community

In soil many microbes live in close proximity, and they interact in a unique way that is in marked contrast to the behaviour of pure cultures.

Members of the microflora rely on one another for certain growth substances but at the same time they exert detrimental influences so that both beneficial and harmful effects are evident (Korf, 1998).

In natural environments, a number of relationships exist between individual cells. The interrelations and interactions of the various microbial groups making up the soil community, however, are in a continual state of change, and this dynamic state is maintained at the level of the flora (Montealegre *et al*., 2002). The composition of the microflora of any habitat is governed by the biological equilibrium created by the associations and interactions of all individuals found in the community. Environmental changes upset the equilibrium but it is re-established, possibly in a modified form as the community shifts to become acclimated to the new circumstances (Korf, 1998).

Microorganisms are considered somewhat volatile regarding species composition and activities. The limited abilities of microorganisms to maintain the environmental conditions surrounding them imply substantial sensitivity of microbial communities to changing environmental factors and biotic interactions such as competition and predation. All these factors play important roles in community dynamics (Piceno *et al*., 2000). Competition between bacterial species for limiting resources has been studied in laboratory systems and also influences bacterial community composition and structure of some natural system (Piceno *et al*., 2000).

Previous studies in the Colorado alpine ecosystem have shown two predictable seasonal patterns in soil microbial biomass. Microbial biomass increases in the fall and winter and decreases in the spring at the start of the plant- growing season. The decline of microbial biomass in the spring has been linked to a pulse of nitrogen availability to plant in the alpine dry meadow. However, the causes of this pattern have not been established. It has been speculated that the decline in microbial biomass in springtime is linked to freeze- thaw events (Lipson *et al*., 2000).

Numerous studies have observed that the freeze- thaw events cause a burst of microbial activity such as mineralization of carbon or nitrogen. This burst of activity has generally been attributed to increased levels of substrate in the soil resulting from damage to microorganisms by freeze-thaw events. However, previous work showed that the soil microbial biomass in alpine dry meadows was not affected by a single freeze- thaw event. It has been shown that rates of freezing and thawing and depth of freeze can have large effects on the survival of microorganisms (Lipson *et al*., 2000).

2.1.3. Environmental influence on microorganisms in nature

Environmental conditions affect the density and composition of bacterial flora and non- biological factors can frequently alter to a great degree the nature of the population and its biochemical potential (Montealegre *et al*., 2002). The environmental variables influencing soil bacteria include moisture, aeration, temperature, organic matter, acidity and inorganic nutrient supply (Atlas, 1984).

Questions regarding microbial composition include: is there a unique microbial community associated with a particular soil type or crop, what environmental factors influence this composition and finally, how

sensitive is community composition to environmental perturbations and human disturbance (Johnson *et al*., 2003).

2.2. Diversity

The classical concept of diversity involves species richness, evenness and composition (i.e. the number of different species present, the relative contribution that individuals of all species make to the total number of organisms present and the type and relative contribution of the particular species present). These have been used to generate commonly used indices of diversity such as the Shannon- Weaver index (H', Shannon and Weaver, 1949).

2.2.1. Richness of microbial species

Richness of microbial species is defined as the number of bacterial species within the community. Chloroform fumigation is widely used to estimate microbial biomass, but it is known that fumigation does not result in a complete kill of all microbes. Typically, 90- 99% is killed but values as low as 37% have been reported. There appears to be no effect of matrix potential or bulk density of soil upon fumigation efficiency. Certain cells may be protected by soil organic matter or their position within the soil matrix. It was hypothesized that fumigation for varying periods of time would provide an experimental means of manipulating microbial diversity. The tenet is that fumigation for increasing periods of time will destroy species that are more chloroform resistant. If the soils were incubated for a length of time post- fumigation, then the resulting microbial communities should be increasingly less species abundant (Insam *et al*., 1997).

2.2.2. Evenness of microbial species

Evenness of microbial species is defined as the relative distribution of individual species within the community. Preliminary results have been obtained from a batch, liquid culture experiment in which a soil suspension was used to ensure as diverse microbial inoculums as possible. These diverse inocula were grown in media of different strengths, Tryptone Soya Broth diluted 10- , 100- , and 1000- fold to ensure no carry over of soil particles and sampled at similar points. It was observed that although total cell numbers varied with the strength of the medium, there was no different in species composition, but an increase in species evenness (as determined from community DNA hybridization in media of increasing strength). So, for these complex communities with the same species composition, a change in the evenness of the species had no effect on function (Insam *et al*., 1997).

The operation of a particular biological system, whether ecosystem or microcosm depends on the interplay of three general abiotic factors: environment, biological community- structure (Diversity) and the biological activity (Function). The role of diversity, particularly of microorganisms and the relationship between microbial communities, particularly in soil are very complex (Torsvik *et al.*, 1990).

Microbial characteristic of soils are being evaluated as sensitive indicators of the soil health because of the clear relationship between microbial diversity, soil and plant quality and ecosystem sustainability. While the understanding of microbial properties such as biomass, activity and diversity are important to scientists in furthering the knowledge of the factors contributing to soil health, results from such analysis may also be useful to extension personnel and farmers in devising practical measures

of soil quality (Hill *et al*., 2000).

2.3. Interactions among species

2.3.1. Neutralism

Neutralism is where no interaction occurs between two microbial populations. It occurs between populations that are usually distant from each other and the two populations behave entirely independently. An example is the marine and lake habitats (Korf, 1998).

2.3.2. Commensalism

Commensalism is an interaction between two populations whereby one population benefits while the other remains unaffected. For example, when a population of the facultative anaerobes utilizes O_2 and the amount of oxygen lowers, it creates habitat for obligate anaerobes (Korf, 1998).

2.3.2. Synergism

Synergism is also being referred to as proto- cooperation. In this interaction, both populations benefit from the relationship, but unlike mutualism, the interaction is not obligate. An example is the conversion of arginine to ornithine by an organism known as *Streptococcus faecalis*, which can be utilized by *Escherischia coli* to produce putrescine. *E. coli* cannot produce putrescine without the help of *S. faecalis*. Therefore, both organisms benefit from putrescine (Korf, 1998).

2.3.4. Mutualism

Mutualism is an interaction among different populations whereby both of

them benefit obligatory. Mutualistic relationships between microbes allow the microbes to act as if they were a single organism with a unique identity. En example is *Cyanobacteria* and fungi resulting in the formation of lichens (Atlas, 1984).

2.3.5. Competition

Competition is a condition in which there is a suppression of organism as the two species struggle for limiting quantities of nutrient, O_2 or other common requirements (Atlas, 1984).

2.3.6. Amensalism

Amensalism is an interaction in which one species is suppressed while the second is not affected typically the result of toxic production. Antibiotics and allelopathy have been used to describe such cases of chemical inhibition (Alexander, 1961).

2.3.7. Parasitism

Parasitism is an interaction that has usually a long period of contact, which may be direct physical or metabolic and the parasite host interaction is quite specific. Viruses are obligate intracellular parasites that exhibit great host cell specificity (Korf, 1998).

2.3.8. Predation

Predation occurs when one organism, the predator, engulf and digest another organism, the prey. For example, *Didinium nasutum* (prey) on *Paramecium* until the *Paramecium* population become extinct (Korf, 1998).

Among biotic factors, predation and competition reduced survival while root growth enhances survival. Clay minerals protect against predation. Other biotic factors that affect survivals include: water tension, organic carbon, inorganic nutrients (N, P), P^H and temperature

2.4. The effect of pollution on soil microbial communities

Although it has been demonstrated that microbial communities can affect chemical pollutants, the presence of chemical pollutants can also affect microbial community structure. The chemical can alter the community structure through selection of pollutant degraders or through acute toxicity to microorganisms. Readily biodegradable pollutants can increase population densities by promoting growth providing carbon and energy to microorganisms. (Long *et al*., 1995). Studies on microbial populations in response to catastrophic oil spills and other exposures to petroleum products in marine and fresh water environments have been made, and results showed that subsurface microbial communities from a pristine aquifer exhibited significant adaptation on exposure to chemical pollutants in microcosm studies (Long *et al*., 1995, Grossman *et al*., 1999).

In soil studies, interactions in the rhizosphere are of the great interest. Through the use of up- to date isotopic techniques, researches gained a great deal of information on the partitioning of assimilated carbon between plant parts and losses of assimilated carbon through rhizodeposition and root respiration. Besides, rhizosphere microbes interactions in form of allelopathy, N- fixation, suppression of soil borne plant pathogenic microbes are also well studied in the study of soil microbial communities (Yevdokimov *et al*.).

Many bacteria in the rhizosphere are stimulated by the presence of the plants. The main source of this stimulation is the availability of complex carbohydrates on plants for accumulation and growth. Plants on the other hand often benefit from the nitrogen compound either fix or released from organic material by bacteria metabolism. For instance, *Azatobacter vinelandii*, a free- living nitrogen fixing bacterium ultimately depends on carbon fixed by plants. While plants growing in low nitrogen soil colonized *A. vinelandii* benefit from the fixed nitrogen compounds (Yevdokimov *et al*.).

Donegan *et al*. (1999) suggested that unintentional changes in plant characteristics resulting from genetic modification might have impact on soil and plant biota. Consequences of these changes include, decrease in plant decomposition rates, and in soil carbon and nitrogen levels that could affect soil fertility. In addition, the root- associated communities of genetically modified plants may be significantly different from non-genetically modified plants (Dunfield *et al*., 2001).

Agricultural chemicals such as pesticides and chemical fertilizers may adversely affect ecosystem if misused. Agricultural chemicals may affect physiology and biochemistry of soil microbes. Previous studies on the effects of agricultural chemicals on soil microbial communities have been mostly focused on soil respiration, soil enzyme activity, soil microbial biomass and soil nitrogen cycling. However, there is an increasing interest in genetic analysis of soil microbial communities at the molecular genetic level (Yang *et al*., 2000).

Pollution of agricultural soils by heavy metals from agricultural and industrial activities is a major environmental problem. Heavy metal pollution cannot only result in adverse effects on various parameters

relating to plant quality and yield, but also cause changes in the size, composition and activity of the microbial community. Abiotic stress caused by heavy metals, in inorganic and organic forms, affects the growth, morphology and metabolism of the microorganisms in soils. Numerous studies have demonstrated the adverse effect of different heavy metals on soil microbial biomass and activity (Yao *et al*., 2003).

Soil microorganisms are sensitive to long term exposure to moderate concentration of heavy metals in soil. Effective strains of *Rhizobium leguminosarum* bv. *Trifoli*, the nitrogen fixing symbiont of white clover (*Trifolium repens* L) were eliminated at metal concentrations too small to cause toxicity to plants. Studies of the population of *rhizobia* at Woburn, UK, demonstrated that only a single isolate of rhizobium survived which was heavy metal tolerant but ineffective in N_2-fixation with white clover. Further research in long-term experiments at Braunschweig, Germany, demonstrated complete loss of *R. leguminosarum* bv. *Trifoli* at metal concentrations within the guidelines for environmental protection in the Europe Union. Therefore, an increase in heavy metal stress results in a decrease in microbial diversity (Lakzian *et al*., 2002).

2.5. Methods for studying microbial communities in soil

By examining how specific microbial groups respond to environmental manipulation, identification of environmental factors that influence the structure of microbial communities and the scale at which the environmental factors influence the distribution of individual microbial groups in the soil should be taken into consideration in determining soil microbial communities (Buckley *et al.*, 2001).

Microbial remediation of toxic hydrocarbon contaminated sites is carried

out by diverse group of microorganisms (Joshi *et al*., 1996). Study of this diversity at the genetic level is necessary to understand the phylogenetic perspective, the mechanism of degradation and develop novel strategies of treatment. Analysis of microbial biodiversity also helps in isolating and identifying new and potential microorganisms having high specificity for recalcitrant compound (Bhattacharya *et al*., 2000).

Documentation of this microbial diversity from oily contaminated sites is essential because they create a major environmental concern and these microbes can be used for cleaning up the environment. Studies by Bhattacharya *et al*. (2000) for the biodegradation of oily contaminated soil have been made and results showed that *Pseudomonas sp*., *Corynebacterium sp*. and *Flavimonas sp*. were the most efficient for biodegradation of the contaminated soil (Bhattacharya *et al*., 2000).
Changes in soil biological community composition might provide a measure of the effects on land use practices on soil health. Although some of these changes can be characterized using microbial isolation procedures. It is estimated that less than 10% of soil microorganisms are culturable using existing techniques. Alternative approaches based upon structural component analysis have been developed to characterized changes in soil community diversity (Dierksen *et al*., 2002).

2.5. PAHs/PCBs

2.5.1. Characteristics

Polycyclic aromatic hydrocarbons (PAH_S) and polychlorinated biphenyl (PCB_S) are chemical compounds that persist in the environment and thus cause environmental pollution worldwide. Their chemical structures, insolubility in water as well as their ability to adsorb to soil components like organic matter are some of the features characterizing their persistence in the environment (Chaineau *et al*., 1997).

The desirable physical and chemical properties of PCB_S (excellent dielectric and flame resistance properties, chemical and thermal stability) led to their extensive industrial use as heat transfer fluids, hydraulic fluids, solvent extenders, plasticizers, flame retardants, organic diluents and dielectric fluids (Abramowicz *et al*., 1995). Polycyclic aromatic hydrocarbons (PAH_S) are non- polar, hydrophobic, neutral molecules with two or more fused benzene rings in linear, step or cluster arrangement (Reilley *et al*., 1996).

2.5.2. Origin

PCB/PAH are pollutants that are widely distributed in the ecosystem by the energy generating and petroleum producing industries. They are widely distributed in oil contaminated soil, ground water and sediments as a result of relatively high aqueous solubility compared to other components of petroleum (Nakagawa *et al*., 2002, http://www.mtsu.edu/~nchong/gc.html). Polychlorinated biphenyls (PCB_S), and polycyclic aromatic hydrocarbons (PAH_S) have been found to exist in soil and sediment partially in a strong sorbed, resistant form and the size of this desorption- resistant fraction may increase dramatically with time as the chemical remains in the soil or sediment (Hatzinger *et al*., 1995).

2.5.3. Formation

In general, PAH_S are formed whenever there is heating of carbon- based material, such as coal, tar, wood and rubber (www.startenvironmental.com). PAH_S occur mostly when there is incomplete combustion of carbon materials (Reilley *et al*., 1996).

2.5.4. Sources

The most significant anthropogenic sources of PAH_S include the heat and power generation from coal and other fossil fuels, coke production, petroleum refining, coal and oil shale conversion and chemically manufacturing (Reilley *et al*., 1996). PAH_S occur as common constituents of petroleum, coal tar and shale oil, but are most frequently formed by incomplete combustion of fossil fuels. Hydrocarbons can reach the soil from many sources including pipeline blowouts, road accidents, leaking of underground, storage tanks, land farming fields and non- controlled landfilling (Maila *et al*., 2002).

2.5.5. Health risks of PAHs/PCBs to humans and animals

Several PAH_S are known to be toxic, mutagenic and carcinogenic. Exposure to PAH_S can lead to a variety of chronic or acute effects in human beings including various forms of cancer and birth effects in newborns. Their elimination is considered a priority in many countries (http://www.ntsu.edu/~nchong/gc.html). PCB_S are suspected of causing a slew of reproductive, neurological and immune disorders in animals and humans. Humans accumulate these organic compounds by consuming seafood such as fish that ingested PCB_S from contaminated fresh water (Reilley *et al*., 1996).

2.5.6. Environmental concern regarding PAHs/ PCBs

Of the different kinds of environmental pollutants, the persistence of PAH_S is of paramount importance

(http://www.mtsu.edu/~nchong/gc.html). The thermal and chemical stability of PAH that led to their widespread industrial use creates a major problem for the disposal of this lipid soluble, toxic xenobiotic with remarkable persistence in the biosphere (Rost *et al*., 2002). One possibility for their removal from the environment is the degradation by microorganisms possessing the appropriate metabolic physiological and kinetic characteristics (Nobbs *et al*., 2003).

The widespread distribution of polychlorinated biphenyls (PCB_S) in the environment was first observed by Jensen in 1966, which demonstrated the presence of this class of compounds in fish taken from Swedish water. The high degree of public concern and anxiety about chlorinated aromatic compounds persisting in the environment mandate that effective clean- up methods be found to eliminate their hazards (Clarke *et al*., 2002). Preliminary studies have shown that low temperature and controlled conditions can achieve reduction of PCB_S in soils and sediments (Nobbs *et al*., 2003, Bolin, 2002).

The increasing use of petroleum products leads to the contamination of the environment. The release of oily waste containing toxic hydrocarbon (HC) has been shown to cause serious damage to natural ecosystem (Chaineau *et al*., 2000). The inadequate use of these oils, lack of proper storage and disposal of spill oils and explosions of transformers cause serious problems in the environments (Avelizapa- Rojas *et al*., 1999).

Since 1929, transformer oils are of environmental concern because of their content in polychlorinated biphenyls (PCB_S) that were widely used during 1929- 1970. During that period 632 millions of PCB_S were produced for several uses. Nearly 26% of the total production was used for transformer oil formulation. The inadequate use of these oils, lack of proper storage and disposal of spent oils and explosions of transformers

caused a serious contamination problem (Avelizapa- Rojas *et al*., 1999).

2.6. Analytical techniques for PAH- relevance

Soil comprises a very complex organo- mineral matrix onto which PAH$_S$ can strongly bind to the organic matter making them very difficult to extract. Although HPLC has been recommended, GC- MS has also been recommended as an alternative method used to identify and to quantify PAH$_S$ and their metabolites (Lavigne, www. dexil com, Binet *et al*., 2001).

2.7. Phytoremediation

For soil contaminated with organic chemicals, remediation techniques such as incineration and landfilling can be expensive. An alternative technique known as phytoremediation, involves plants to clean up the environment (Nichols *et al*., 1997).

Phytoremediation is a technique that is used to clean- up the environment contaminated with hazardous pollutants. Plants remediate organic pollutants by: 1) direct up- take of contaminants, their conversion and accumulation of non- phytotoxic metabolites. 2) release of exudates and enzyme stimulating microbial activity and biochemical transformation. 3). enhancement of mineralization in the rhizosphere (Mackova *et al*., 1997). Rhizoremediation is an effective, non- disruptive and inexpensive mean of remediating environments. (Chaney *et al*., 1997).

It is more cost effective than alternative mechanical and chemical methods of removing hazardous compounds from the environments (Pradhan *et al*., 1998). Besides, rhizoremediation is a natural, aesthetically pleasing low cost technology. It is socially accepted by sorrounding communities and regulatory agencies as a potential elegant and beautiful

technology (Alkorta *et al*., 2001).

2.7.1. Advantages of phytoremediation

Rhizoremediation has made tremendous gains in market acceptance in recent years. In addition to its favourable economics, the main advantages of rhizoremediation in comparison with classical remediation methods can be summarized as follows:

- It is far less disruptive to the environmental
- There is no need for disposal sites
- It has a high probability of public acceptance
- It avoids excavation and heavy traffic
- It has potential versatility to treat a diverse range of hazardous materials (Alkorta *et al*., 2001).

2.7.2. Disadvantages of phytoremediation

Like other methods of environmental remediation, rhizoremediation has its disadvantages. The use of rhizoremediation is limited by the climatic and geological conditions of the site to be cleaned, temperature, altitude, soil type and accessibility by agricultural equipment. The main concerns come from the following problems:

- Formation of vegetation may be limited by extremes of environmental toxicity
- Contaminants collected in leaves can be released again to the environment during litter fall
- Contaminants can be accumulated in fuel woods
- The solubility of some contaminants may be increased resulting in greater environmental damage/ pollutant migration (Alkorta *et al*.,

2001).

All plants modify the surrounding soil (rhizosphere) through the release of organic and inorganic substrates. Roots receive 30-60% of the net photosynthetic carbon, from which 10-20% is released by rhizodeposition. Exudates consist primarily of low molecular weight (LMW) and high molecular weight (HMW) organic acids. The exudates cause rhizosphere-inhibiting microbial populations to increase well beyond those of the bulk soil, attracting motile bacteria and fungal hyphae that stimulate an array of positive, neutral and / or negative interactions with plants (Singer *et al*., 2003).

The concept of using plants to remediate soils contaminated with organic pollutants is based on observations that disappearance of organic chemicals is accelerated in vegetated soils compared with surrounding non-vegetated bulk soils (Li *et al*., 2002). Phytoremediation of organic contaminants has generally focused on three classes: i.e. Chlorinated solvents, explosives and petroleum hydrocarbons. Plants that are less affected by compounds in contaminated soils are healthier and more persistent and will produce healthier root systems and greater top growth (Alkorta *et al*., 2001).

The chemical composition of root exudates and rates of exudation differ among plant species. This has led some research group to screen for plant species that exude phenols capable of supporting PCB-degrading bacteria. Since not all plants produce and release the same types of phenolics, it would be expected that some plant may preferentially habour PCB-degrading in the rhizosphere (Alkorta *et al*., 2001).

Fletcher and Hedge (1995) screened 17 different perennial plants for release of phenol that could support PCB-degrading microbes and found that mulberry (*Morus rubra* L) had many attributes that would favour its use in phytoremediation. Rhizospheric microorganisms may also accelerate remediation process by volatizing organics such as PAH_S or by increasing the production of humic substances from organic pollutants (Dunfield *et al*., 2001).

In addition to secreting organic compounds that support the growth and activities of rhizosphere microorganisms, plants also release a number of enzymes into soils and water and these enzymes degrade organic contaminants. Soil enzymes derived from plant sources include: laccases, dehalogenase, nitoreductase, nitrilases and peroxidases. Field tests of plant- derived nitroreductases and laccases showed significant degradation of ammunition wastes (TNT, dinitromonoaminotoulene and mononitrodiaminotoulene) and triaminotoulene, respectively. Similarly, other studies have examined the ability of a nitrilase to degrade 4-chlorobenzonitrile and of halogenases to metabolize hexachloroethane (Burken *et al*., 1996).

2.8. Direct uptake of organic contaminants

Bioavailability of organics in soil appears to be a primary restriction for effective phytoremediation of organic pollutants. The use of synthetic (Triton X-100, SDS) and naturally produced biosurfactants (rhamnolipid) to enhance the apparent water solubility and bacterial degradation of organic contaminants is well documented in the study of phytoremediation (Watanabe *et al*., 2001).

Potential advantages of using biosurfactants or cyclodextrins, which have the ability to solubilize both organics and metals, could be instrumental in remediation of soil with mixed contaminants. If the plants are to be used for phytoextraction of organic contaminants, it is essential to determine the fate of the parent compounds and their metabolites. Following uptake, organic compounds may have multiple fates: they may be translocated to other plant tissues and subsequently volatilized, they may undergo partial or complete degradation or they may be transformed to less toxic compounds and bound in plant tissues to non- available forms (Burken *et al*., 1996).

Soil environments influenced by plant roots or rhizosphere often has significant different properties than the bulk soil. Plant exudation of soluble organic and inorganic compounds provides a substrate for microbiological growth and may interact directly with the soil solids. Organic carbon, P^H, biological activity and the solubility of inorganic constituents are significantly altered in the rhizosphere (Reilley *et al*., 1996).

2.9. Rhizosphere microbial communities

2.9.1. The role of rhizosphere microbial communities in bioremediation

Plants can accelerate bioremediation in surface soils by stimulating the growth and metabolism of soil microbes through the release of nutrients and the transport of oxygen to their roots. The zone of soil closely associated with the plant root, the rhizosphere, has much higher numbers of metabolically active microbes than the surrounding bulk soil (Li *et al*., 2002). Plant roots release compounds including simple sugars, amino acids, enzymes, aliphatics and aromatics that encourage the growth of specific microbial community (Burken *et al*., 1996).

The interactions between plants and microbes in the rhizosphere are complex and in some cases have evolved to the mutual benefit of both organisms (Macek *et al*., 2000). The sorption capacity of soil depends upon the quality and quantity of organic matter and clay minerals and therefore varies with soil type. The aged or desorption resistant fraction of organic compounds may result from the slow diffusion of these molecules within some components of soil organic matter in soil. A second hypothesis for aging suggest that chemicals slowly diffuse into and become entrapped within small pores in soil aggregates (Hatzinger *et al*., 1995).

The mutualistic relationship is responsible for the accelerated degradation of soil contaminants in the presence of plants. In addition to this rhizosphere effect, plants themselves are able to take up a wide range of organic wastes from soil or water through the roots (Macek *et al*. 2000). Increases in microbial numbers and activity in vegetated soil as compared to non- vegetated soil are largely due to organic and nutrients additions by plants and the distribution of organic matter present in the soil environment (Burken *et al*., 1996). Plants that derived compounds such as flavonoids could support the growth of some PCB- degrading microbes and enhance PCB metabolism, by providing the first alternative to biphenyl (Schnelle *et al*., 2003).

2.10. Rhizoremediation

The presence of decomposing organic matter (unrefined), fossil fuel and volcanism continually produce a wide range of chemicals (e.g. polycyclic aromatic hydrocarbon) referred to as persistent organic pollutants when of anthropogenic origin (Singer *et al*., 2003) (www.smartenvironmental.com). Different mechanisms have been proposed to explain the effect of plant rhizosphere on PAH dissipation

such as an increase in microbial numbers, an improvement in the physical and chemical soil conditions, increased humification and adsorption of pollutants in the rhizosphere. However, the relative contribution of each process has not been clearly elucidated (Binet *et al*., 2001).

Over the past 20 years, numerous studies have reported the capacity of various bacteria, fungi and algae to degrade polycyclic aromatic hydrocarbons (PAH_S) and polychlorinated biphenyls (PCB_S) (Haluska *et al*., 1995). However, few studies have focused on the contribution of individual microorganisms in reducing the toxicity of PAH/PCB compounds. Microbial degradation of PAH_S may result in the incomplete breakdown of the compound depending on the environmental conditions and the microbial population present (Juhasz *et al*., 2000).

Once a soil has been contaminated with PAH_S the major pathways include volatilization, sorption, leaching, and accumulation by plants and biodegradation. The ultimate fate of PAH_S in the soil is controlled by surface adsorption. Soil bacteria are the primary degraders of PAH_S in soil with *Pseudomonas, Mycobacterium, Flavobacterium, Acinetobacter, Arthrobacter*, *Bacillus* and *Nocardia* being the most active species (Reilley *et al*., 1996, Daane *et al*., 2001).

Biodegradation of PAH_S by soil microorganisms has been identified as a major mechanism for bioremediation of contaminated soil (Binet *et al*., 2001). Many bacterial strains have been isolated for their ability to degrade and use PAH_S as a source of carbon and energy (Dagner *et al*., 1997).

Roots of some plant species enhance the degradation of recalcitrant, organic soil contaminants (i.e. PCB and PAH) by releasing cometabolites and facilitating aeration. Purified natural plant compounds (i.e. flavonoids)

stimulate the growth and activity of PCB and PAH degrading bacteria (Fletcher *et al*., 1995, Daane *et al*., 2001). Soil associated with inappropriate maneuvering of field equipment and / or modern cropping system negatively affect soil physical properties and thus may limit microbial activities and biochemical processes, which are important to nutrient bioavailability (Li *et al*., 2002).

The extreme persistence of these aromatic hydrocarbons in the environment led to the early notion of the incapability of indigenous microorganisms to degrade and recycle these compounds in nature. Later on, isolation and characterization of a variety of microbes from the environmental samples capable of metabolizing aerobically PCB_S has incited interest in using biodegradation for the recycling of these xenobiotics (Damaj *et al*., 1995).
The fate of xenobiotics in soil depends not only on static properties, but also on dynamic parameters, such as water flow and diffusion of solutes. PCB and PAH as hydrophobic compounds strongly sorbs to soil particles and only the fractions dissolved in water, is available to mast microbial system for biocatalytic transformation (Vrana *et al*., 1995).

Several studies dealing with the biodegradation of PCB by microorganisms have been published and demonstrated that; the biodegradative capacity of interaction populations of bacteria must be considered in order to gain a better understanding of PCB degradation in the environment (Haluska *et al*., 1995). A positive effect of mineral salts and nutrients as well as of biphenyl as sole carbon source on the mineralization of PCB in aquatic sediments and spiked soil has been observed. Biodegradation of Arochlor spiked soil has been demonstrated with resulting cells of *Pseudomonas putida* grown on biphenyls (Fiebig *et al*., 1993).

When comparing the performance of the PCB-degrading bacteria strains in

liquid media and in soil, the degradation was less efficient in soil microcosms and further reduction of efficacy was observed under environmental conditions (Zachar *et al*., 1996). Biodegradation is associated with soil conditions that are affected by a whole host of interrelated physical, chemical and biological and environmental processes (Vrana *et al*., 1995).

Since *Rhizobia* by definition and classification, are soil bacteria associated with the roots of plants and rhizosphere, they are naturally exposed to a range of aromatic exudates of roots and thus may prove to possess interesting aromatic catabolic pathways and capabilities. However, to our knowledge, they have not yet been considered and studied for use in bioremediation of aromatic pollutants, perhaps because the focus always been oriented toward their N_2 fixing features (Damaj *et al*. 1995).

The degradation rates are dependent on the kinetic parameters of the inoculated strains that can be influence by both environmental factors (soil p^H aeration, nutrient and moisture) and microbial factors (quality and amount of indigenous microflora) in conjunction with the bioavailability of the pollutant. Among the physical and the chemical factors, organic matter content, presence of alternative carbon sources and bioavailability of the pollutant may largely impair the survival of microcosms and their ability to degrade the substrate (Vrana *et al*., 1995).

It is known that microbes in rhizosphere soil are capable of degrading a variety of contaminants. Furthermore, inoculation of plants with selected

bacteria can increase rhizoremediation activity (Siciliano *et al*., 1999).

PCP (Pentachlorophenol) is a serious contaminant in surface soils, and is susceptible to mineralization by a few strains of bacteria (Pfender, 1996). When PCP is present at a low concentrations (≤ 10mg/kg), microorganisms especially PCP degrading microbes become resistant to PCP contamination, however, when the concentration is higher, PCP degrading microorganisms are being inhibited by the toxicity of PCP (Pfender, 1996; Steiert et al., 1989).

Studies have shown that certain plants decrease amount of 2-chlorobenzoic acid (2- CBA) in soil and that inoculation of these plants with specific bacterial seed inoculants increased 2- CBA degradation (Siciliano *et al*., 1999). For example, growth of Dahurian wild rye (*Elymus dauricus*) doubled 2CBA degradation compared to bulk soil and amounts of 2CBA were further reduced by inoculating seed with *Pseudomonas aeruginosa* strain R75, *P.savastanoi* strain CB35 or a mixture of these strains. Similarly, other studies shown that inoculation of bean (*Phaseolus vulgaris*) with *Pseudomonas fluorescens* 2- 79 RLD increased the rate of 2, 5- dichlorobenzoic acid (2,5diCBA) degradation. Furthermore, inoculation of corn with a consortium of *Clavibacter michiganese*, *Pseudomonas sp*. and *Cytophaga sp*. Increased atrazine mineralization (Siciliano *et al*., 1999).

Because a sufficient accessibility and utilization of organic xenobiotic compounds promote both the evolution of complete degradative pathways in single bacterium and the natural enrichment of pollutant- degrading microorganisms, the specific abilities of the autochthonous microbiota can be taken as an indication of success of bioremediation. Further more, the percentage of pollutant- degrading organisms in a microbial community is

a decisive criterion for the efficiency of biological decontamination (Becker *et al*., 1995).

While numbers of pollutant-degrading isolates in themselves are of little value for a comparison of different sites or conditions, determination of cell counts as relative proportion in the entire communities provided both determination of the percentage of pollutant-degrading microorganisms and the assessment of bacteria that are incapable of xenobiotic use but compete for bioelements (Becker *et al*., 1995).

Aquifer microbial communities can degrade a broad range of naturally occurring and xenobiotic compounds under a broad range of environmental conditions. Studies have been made of the research of subsurface microbial ecology and the results obtained showed that indigenous aquifer microbial populations could biodegrade environmental pollution and affect remediation of contaminated sites (Long *et al*., 1995).

2.11. *Pseudomonas* as a PAH-degrading bacterium

Bacteria of the genus *Pseudomonas* are ubiquitous and have been isolated from many habitats including different soil types, fresh and marine waters, plant leaves, plant roots animal skin and animal tissues (Dunfield *et al*., 2001). The ability of *Pseudomonas* to colonize a wide variety of habitats resides not only in the capability to adhere to solid particles and their motility, but also in their metabolic versatility which allows them to use many natural and xenobiotic compounds as C-, N-, S- and P- sources. *Pseudomonas putida* mt-2 is soils isolate which utilizes toluene, xylenes and alkylbenzoate as sole C- sources because it possesses the TOL plasmid, PWWO (Molina *et al*., 2000). A bacterium known as *Rhodococcus erythropolis* TA421, a gram-positive bacterium isolated from dry wood termite ecosystem has been shown to easily degrade several highly

persistent xenobiotics such as PCB_S (Schnelle *et al*., 2003).

2.12. Methods to study diversity and function of microbial communities

2.12.1. Biolog System

The lack of knowledge about the diversity and function of microbial communities is due to the fact that a need exist for effective methods to evaluate microbial community structure. Isolate- based techniques have often been used, but they offer a limited, biased view of microbial communities due to 1) selective nature of laboratory media, 2) the unculturability of many microorganisms, 3) the multiple microbial interactions and the difficulties of determining microbial function in situ (Korf, 1998; Gerland, 1997).

The rRNA approach together with other molecular techniques and biochemical techniques such as signature lipid biomarker analysis enables identification and phylogenetic characterization of microorganisms without cultivation. However, these methods are time consuming, complex and it is difficult to interpret the data obtained from these techniques regarding diversity of microbial communities. Hence, there is a need for techniques that allow one to characterize microbial communities without the usual reliance on selective culturing and which would be less complex than the molecular techniques (Korf, 1998; Ellis *et al*., 2001).

Complete cataloguing and quantification of indigenous bacteria species could be the ultimate aim of microbial community analysis. However, there is a need for rapid, simple and cost saving techniques that do not necessarily identify all the individual species present in the habitat but can differentiate microbial communities either in terms of structure or function (Verschuere *et al*., 1997). Over the past 10 years the approach to analyzing soil microbial communities has changed dramatically. New

methods and approaches are know available, allowing soil microbiologists to gain access to more of the microorganisms residing in soil and allowing for better assessments of microbial diversity (Hill *et al*., 2000).

In 1991, a method was published for the functional characterization of microbial communities using commercially available (BIOLOG GN) 96 well plates containing 95 different carbon sources and a dye (tetrazolium violet) that is reduced by microbial metabolic activity. Gerland *et al*., (1991) introduced the biolog system (Biolog Inc., CA, USA) as a rapid community level tool to characterize and classify heterotrophic microbial communities based on sole carbon source utilization pattern. Microtiter plates are commercially available that contain a large number of metabolic tests. The biolog system is based on 95 individual carbon source oxidation tests, for which the colour change of the redox dye tetrazolium violet is used as an indicator of consumption of the sole carbon source (Gerland, 1997).

Bacteria communities can be described by their enzymatic potentials using the biolog substrate utilization assay to assess the impact of e.g. use of xenobiotic compounds or released bacteria on the soil environment. It is important to be able to monitor changes in the diversity and activity of bacterial communities. Further, in ecological studies, easy and reliable techniques to describe the structure and dynamics of bacteria communities are needed (Insam *et al*., 1997).

The Biolog substrate utilization assay for the identification of bacteria has been used to characterize microbial communities. For instance, it has been possible to differentiate between microbial communities from fresh water, rhizosphere and nutrient- enriched soil, from agricultural and beech forest soil (Gerland *et al*., 1991), plant communities and microbial communities extracted from different soil aggregates, although Haack *et al*. (1995) were

unable to detect differences between rhizosphere and bulk soil microbial communities. Furthermore, the assay has been able to monitor effects of different biotic treatments of soil.

The main virtue of this substrate utilization assay is its ease of use, making it practical for thoroughly replicated large-scale studies. This assay has been used to study microbial communities in a variety of ecosystem (Buyer *et al*., 2001; Van Heerden *et al*., 2002).

Simplicity of the method and the commercial availability of the microtiter plates make this approach very attractive. Consequently, the biolog system has been used to characterize microbial communities of soil, water, plant rhizosphere and phyllosphere microbial communities, activated sludge and composted manure (Verschuere *et al*., 1997, O'Connell *et al*., 2002; Thantsha, 2002).

2.12.2. Sample treatment from collection to incubation

Collecting environmental samples inevitably cause disturbance of the physical, chemical and biotic components. Thus, from the moment a sample is taken, the community within it is likely to be changing due to death of some individuals or populations, expansion of certain opportunistic species, and the use of phenotypic mechanisms to adapt to the altered environment. Time from sample collection to inoculation of Biolog plates should therefore be kept to a minimum, preferably within the same day. Soil storage, even in cold or frozen is not recommended as bacterial, actinomycete and fungal populations can be reduced and affected (Preston-Mafham *et al*., 2002).

2.12.3. Factors influencing the Biolog plates

Biolog plates have thus been proven useful as a metabolic fingerprint of bacteria communities (Yan *et al.,* 2000). However, to interpret the biolog fingerprint, it is essential to know how different inoculum sizes and incubation conditions influence the tetrazolium reduction. Inoculation of bacteria in the biolog plates is expected to result in growth, and the rate of formazan development in individual wells has been assumed to represent growth of the inoculated bacteria and to correlate positively with inoculum cell density (Gerland *et al*., 1991).

A) Incubation condition

Most bacterial Biolog investigations have used fixed incubation temperatures between 15 and 28 ^{0}C. However, when seasonal changes to a community are being investigated, the appropriate environmental temperature for that time of year may be key to determining the functional response of the community. The Biolog approach is sensitive to O_2 concentration. This is indicated by the fact that under anaerobic conditions no formazan is produced and that since the density of actively respiring cells is better correlated with rate of colour development than is total density, indicating that the physiological state of cell can influence rate of colour development. Thus, the distribution of O_2 throughout the wells is important to limit errors (Proston- Mufham *et al*., 2002).

B) Inoculum cell density

There are a number of important considerations in the use of community-level physiological profiles method for community analysis. First, the density of the initial inoculum must be standardized because it affects the rate at which colour develops in the wells and the time at which colour development should be measured. Visible colour will not develop within a well until the total number of cells able to utilize that substrate reaches approximately 10^8 cells/ ml. Because the number of cells directly

inoculated into the wells may be below 10^8 cells/ ml, there can be a substantial lag phase while the cells grow within the well. This may lead to false negatives if wells are read too soon. Inaccurate physiological profiles may also result if samples are dominated by only a few species capable of growing on particular substrates (Hill *et al*., 2000).

C) Ecological relevance of the substrates

Another problem is that the substrates found in the commercially available Biolog plates are not necessarily ecological relevant and most likely do not reflect the diversity of substrates found in the environment. Studies have been made in which plant root exudates compound were included as carbon sources in a functional analysis of nine upland grassland sites. The carbon sources most useful in differentiating the different sites were predominantly these plant root exudates. All of these compounds had low utilization rates suggesting they were utilized by microorganisms that were present in the soil in low number (Hill *et al*., 2000).

2.13. Statistical analysis of Biolog data

Biolog data are well suited for multivariate statistical analysis such as principal component analysis and cluster analysis, tools that can distinguish among bacterial communities from various environments and can be used to describe temporal changes in physiological characteristics (Choi *et al*., 1999). Most commonly multivariate statistical techniques are necessary to analyze the substrate utilization profile data. For example, community are considered to be functionally similar if the utilization profile of the 95 different carbon sources from one community clusters with that from another community. If the profiles segregate, community would be considered functionally different. As such, community- level

physiological profiles can be useful in assessing gross functional diversity (Hill *et al*., 2000).

2.14. Incubation time effects on the Biolog System

Furthermore, the period of microbial growth within the well may also lead to competition effects that again may bias the substrate utilization profile. Perhaps the best way to standardize inoculum levels is to employ vital stains combined with epifluorescence microscopy as a means to quantify actively respiring cells. This way, a standard population of metabolically active cells can be introduced into each well (Hill *et al*., 2000).

2.15. Factors that influence colour development

Functional diversity is based on the assumption that colour development in each well is solely a function of the proportion of organisms present in the sample, which are able to utilize a particular substrate. However, this may not be valid given that some strains may utilize certain substrates more efficiently than others predominating in the well and resulting in proportions of strains that differ from the original sample. Furthermore, the ability of different taxa in the sample to utilize the same carbon sources is generally unknown (Hill *et al*., 2000).

2.16. Genetic methods

The genetic complexity or genome size of microbial community genomes can be assessed by re-association of community DNA. Such broad scale analysis has revealed that the community genome size equals the size of 6000-10000 *Escherischia coli* genomes in unperturbed organic soils, and 350-1500 genomes in aerable or heavy metal polluted soils (Torsvik *et al*., 2002; Buckley *et al*., 2001).).

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A number of methods with higher resolution power have been developed for characterization of microbial communities that includes both cultured and uncultured microorganisms. Most studies are based on the application of the 16S rRNA as a phylogenetic marker to describe the diversity, richness and structure of bacterial populations (Sessitsch *et al*., 2002; McCaig *et al*., 2001).

2.16.1. RNA

The relative abundance of microbial groups is determined by extracting total RNA from soil and challenging the extracted RNA with oligonucleotide probes specific for RNA from the alpha, beta and gamma *Proteobacteria*, the *Actinobacteria* (Gram positive bacteria with high mol. % G+C content), the bacteria and the eukarya. Results obtained found that of all bacteria groups surveyed, the alpha *Probacteria* composed of the largest fraction of community rRNA whereas the *Actinobacteria* were the second most abundant group surveyed, followed by the beta *Proteobacteria* and the gamma *Proteobacteria* (Buckley *et al*., 2001).

Recently, cultivation independent approaches utilizing 16S rRNA have been used to explore the taxonomic diversity of soil microbial communities. These 16S rRNA-based techniques can also be exploited to examine the distribution of specific microbial groups in relation to the environmental characteristics (Smith *et al*., 2001).

2.16.2. PCR techniques

In general, environmental DNA is used as a template for PCR amplification of bacterial 16S RNA genes and subsequent community analysis detects bacteria irrespective of their viability or metabolic activity (Sessistsch *et al*., 2002, Torsvik *et al*., 2002). Polymerase Chain Reaction (PCR) techniques such as Random Amplified Polymorphism DNA (RAPD) or Denaturant Gradient Gel Electrophoresis (DGGE), gives information on species composition and can be used to compare the common species present in samples (Dierksen *et al*., 2002, Cho *et al*., 1996). The use of volatile organic compounds (VOC'S) to obtain a functional profile, analogous to PLFA analysis, is another potential indicator for functional diversity (Dierksen *et al*., 2002).

However, the extraction of DNA from different solid and heterogeneous materials is not simple because of the humic substances found in the soils (Torsvik *et al*., 1996). The critical phase for soils samples is separation of DNA from humic substances since both of these are acid macromolecules. Humic substances interfere with PCR (Niemi *et al*., 2001). Krsek and Wellington (1999) observed that proteins as impurities of soils DNA extract also caused inhibition of PCR.

2.16.2.1. Random amplification polymorphic DNA

Random amplification polymorphic DNA (RAPD) has been used in species classification and phylogenetic analysis, resistant gene identification, genome analysis and genetic analysis of populations and it is quick, simple and inexpensive. In fact, RAPD analysis has become one of the most popular DNA based methods for assessing genetic diversity in plants and has been used in DNA analysis of soil microbial communities (Yang *et al*., 2000).

2.16.2.2. DGGE, T-RFLP and ITS

Other approaches for fingerprinting microbial communities are DNA-based methods such as Denaturant Gradient Gel Electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and intergenic transcribed spacer (ITS) analysis. These methods are based on the analysis of nucleic acid sequences, rather than membrane components and provide the first step for a more targeted and detailed analysis of composition and diversity (Johnson *et al*., 2003).

2.16.2.3. DNA reassociation

Diversities in bacterial communities are normally determined by phenotypic characterization of isolated strains. DNA can be isolated from bacterial fractions containing 50-80% of the soil bacteria and may provide genetic information about the non-culturable bacteria in soil. The heterogeneity of this DNA is a measure of the total number of genetically different bacteria in soil. Thermal denaturation and reassociation can determine DNA heterogeneity (Torsvik *et al*., 1999).

The non-cultivability of environmental species is well established, and analysis of environmental DNA is being used to overcome this. While the

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DNA reassociation of prokaryotic DNA has been used to estimate the richness of bacterial species (Wagner *et al*., 1993), DNA cross-hybridization gives a measure of the DNA that is common between sample (i.e. the similarity of DNA which is a measure of the relative species composition) diversity and also a measure of the relative diversity of the sample (Torsvik *et al*., 1999; Lee *et al*., 1990).

2.16.2.4. Recombinant DNA

Recombinant microorganisms have been produced that possess improved efficacy and range as plant symbionts and biological control agents and that can produce novel compounds. Because some recombinant microorganisms and transgenic plants carry genes and produce compounds foreign to their environment, can grow and establish outside of their natural habitat and have enhanced survival, persistence and competitive capabilities (Donegan *et al*., 1999).

2.17. Fatty acid methyl ether (FAME)

The carbon utilization and Fatty Acid Methyl Ester (FAME) profiles of the microbial community associated with the roots of the field grown genetically modified canola variety, quest (*Brassica napus*) were significant different from the profiles of communities associated with two non-genetically modified varieties, exel (*Brassica napus*) and parkland (*Brassica rapa*) (Dunfield *et al*., 2001).

2.18. Phospholipid fatty acid (PLFA) analysis

Progress in describing agricultural soil communities has been made using Phospholipid fatty acid (PLFA) analysis to identify statistical relationship

between agricultural microbial communities and crop, soil type and management practices (Widmer *et al*., 2001). The PLFA method provides quantitative data, which is useful for screening a large number of samples and can detect both short and long changes in microbial community structure (Frostergard *et al*., 1993).

The diversity component cannot distinguish between species richness and evenness. The interpretation of the results is also dependant on the %G+C content of the DNA. A shift in the %G+C content can be use to determine changes in microbial community structure, but does not reveal any of the diversity parameters (richness, evenness and composition). The same is true of Phospholipid Fatty Acid (PLFA) analysis, an alternative approach that also overcomes the problem of non- cultivability (Frostegard *et al*., 1993).

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Chapter 3

Microbial diversity in the rhizosphere of selected plants growing in hydrocarbon contaminated and uncontaminated soil.

3.1. Abstract

Diversity and dynamics of microbial communities have been analysed by cultured- dependent methods, which exclude the majority of fastidious microbes due to the selective media. Molecular techniques have been used to determine the diversity of microbial communities. An alternative approach is to examine the components of functional biodiversity in terms of the pattern of substrate utilization. The objective of this research was to study the microbial diversity in the rhizosphere of selected plants grown in hydrocarbon contaminated and uncontaminated soil. Different substrate profiles were generated by inoculating Ecoplates microtiter plate wells with different dilutions (10^{-2}, 10^{-4} and 10^{-6}) of hydrocarbon contaminated and uncontaminated rhizosphere soil of *Elusine coracana* and *Biddens pilosa*. Biolog results indicated that the incubation periods of 24 and 48h were not sufficient to complete colour development in the Ecoplate microtiter plates. Complete colour development was indicated after incubation periods of 96h. The species diversity in the uncontaminated rhizosphere was high but unevenly distributed whereas the diversity in the contaminated rhizosphere was lower and evenly distributed indicating predominance of certain bacteria.

Key Words: microbial diversity, microbial communities, Ecoplates, *Elusine coracana*, *Biddens pilosa*, Biolog, rhizosphere, soil, hydrocarbon.

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3.2. Introduction

For soils contaminated with organic chemicals, remediation technology such as incineration and land filling can be expensive. An alternative technology known as rhizoremediation, involves the use of microorganisms to break down contaminants. Microorganisms can use the contaminants as energy and carbon sources while transforming them into carbon dioxide and water (Nichols *et al*., 1997).

Microorganisms can also transform certain organic compounds while not deriving energy or carbons in a process known as co-metabolism. The area around plant roots, known as the rhizosphere contains higher populations and greater diversity of microorganisms than soil with no plants (Nichols *et al*., 1997).

Rhizoremediation is an emerging green technology that uses plants to remediate soil, sediment, surface water and ground water environments contaminated with toxic metals, organics and radio nuclides. This technology is an effective, non- intrusive and inexpensive means of remediating soils. It is more cost effective than alternative mechanical or chemical methods of removing hazardous compounds from the soil (Alkorta *et al*., 2001; Chaney *et al*., 1997). Besides, rhizoremediation is a natural, aesthetically pleasing low cost technology. It is socially accepted by surrounding communities and regulatory agencies as a potentially elegant and beautiful technology (Alkorta *et al*., 2001).

Plants may enhance remediation of contaminated soil because they function like a solar driven pump that has degradative activity in the rhizosphere. The more soluble the organic compound, presumably the greater is the probability that plants will reduce the concentration (Liste *et al*., 2000). Plant root exudates not only provide the essential resources to sustain the abundant and diverse rhizosphere microbial community, but also contain the chemical signals, which influence interactions in the rhizosphere (http: www.bbsrc.ac.uk/science/initiative/bire.htm). Plants release exudates into the soil ecosystem that increase microbial activity and aid the degradation of xenobiotic organics. The soluble root exudates include enzymes, aliphatics, aromatics, amino acids, sugars and low molecular weight carbohydrates (Burken *et al*., 1996).

The plant rhizosphere is traditionally recognised as a niche rich in growth substrates in comparison with the surrounding bulk soil (Dunfield *et al.*, 2001). In rhizoremediation, plant roots sustain the degrading microflora by supplying it with nutrients other than pollutants, and also help spreading the degrading microorganisms to new sites in the soil (http://www.nessling.fi/symposiot/symposio2/lindstrom.htm).

While analytical methods have been developed for estimating the bioavailability of metals in soil, the relationship of these values to ecological toxicity is not fully understood. Therefore, the true indicators

of the ecological harm caused by contaminants will be the indigenous organisms. Of these, microbes are the most obvious group to study, as they are ubiquitous, respond rapidly to changing conditions and it has been suggested that they should be included in ecological risk assessments (Ellis *et al*., 2001).

Microbial characteristics of soils are being evaluated increasingly as sensitive indicators of soil health because of the clear relationships between microbial diversity, soil and plant quality and ecosystem sustainability (Hill *et al*., 2000).

Abiotic stress caused by heavy metals in inorganic and organic forms, affects the growth, morphology and metabolism of the microorganisms in the soil (Reilley *et al*., 1996). More recently, microbial community structure has also been recommended as a biological indicator of heavy metal stress (Yao *et al*., 2003). The increasing use of petroleum products leads to the contamination of the environment. Hydrocarbons can reach the soil from many sources including pipeline blowouts, road accidents, leaking of underground storage tanks, land farming fields and non- controlled land filling. The release of oily waste containing toxic hydrocarbons (HC) has been shown to cause serious damage to natural ecosystem (Chaineau *et al*., 2000). The inadequate use of these oils, lack of proper storage and disposal of spent oils and explosions of transformers cause serious problems in the environments (Avelizapa- Rojas *et al*., 1999).

Microbial diversity in natural environments has been studied traditionally by the cultivation of bacteria on laboratory media and the subsequent characterization of pure isolates. This approach has several limitations, including selective enrichment of bacteria on the media and under the cultivation conditions used, and the inability to recover starving, dormant and viable but non- culturable cells (McCaig *et al*., 2001).

Advances in molecular ecology, in particular development of 16S rRNA-based methodologies, enables cultivation-independent analysis of bacterial community composition through the detection and characterization of bacterial nucleic acid sequences within environmental samples (Theron *et al*., 2000). The most common approach involves polymerase chain reaction (PCR) amplification, cloning and sequence analysis of the 16S rRNA genes and has been used to investigate the diversity and community composition of a range of terrestrial environments (McCaig *et al*., 2001; Niemi *et al*., 2001). But the extraction of DNA from different solid and heterogenous materials is not simple especially in soil environments because of the humic substances that inhibit microbial DNA (Niemi *et al*., 2001).

However, molecular techniques and biochemical techniques such as signature lipid biomarker analysis are furthermore time consuming, complex and it is difficult to interpret the data obtained from these techniques regarding diversity of microbial communities (Korf, 1998). Therefore, there is a need for rapid and simple techniques that do not necessarily identify all the individual species present in the habitat but that can differentiate microbial communities either in terms of structure or function (Verschuere *et al*., 1997).
Gerland and Mills (1991) introduced a community level physiological approach with Biolog GN microtiter plates for characterizing microbial communities. Since then, several authors have applied this method successfully in their field of research (Hitzl *et al*., 1997; Van Heerden *et al*., 2002).

Microtiter plates are commercially available that contain a large number and variety of metabolic tests. The biolog system is based on 95 individual carbon source oxidation tests, for which the colour change of

the redox dye tetrazolium violet is used as an indicator of consumption of the substrates (Gerland, 1997). The simplicity of the method and the commercial availability of the microtiter plates make this approach very attractive. Consequently, the biolog system has been used to characterize microbial communities of soil, water, plant rhizosphere and phyllosphere microbial communities, activated sludge and compost manure (Verschuere *et al*., 1997; Van Heerden *et al*., 2002).

The hypothesis is that the more substrates utilized, the higher the diversity due to the collective action of individual species. Hence, high diversity was expected in uncontaminated rhizosphere soil of *E. coracana* and *B. pilosa* and low diversity in hydrocarbon contaminated rhizosphere soils of both plant species.

The objective of this research was to study the microbial community structure in the rhizosphere of selected plants growing in hydrocarbon contaminated and uncontaminated soil.

3.3. Materials and methods

3.3.1. Soil sampling and site description

Uncontaminated soils were collected from the agricultural farm of the University of Pretoria, S.A. and the hydrocarbon- contaminated soils were taken from an industrial polluted site in close proximity of a petrochemical plant in Gauteng Province, S.A.

3.3.2. Plants

Seeds of *Elusine coracana* and *Biddens pilosa* were used in this study. These

plant species were selected in this study because of their effective and extensive root system and also because of their easily adaptation in change in environment. They were harvested from the agricultural farm of the University of Pretoria, Pretoria, S.A during winter season and kept in a green house in sterile plastic bags until used. Seeds were sown in four separate trays, each tray containing twenty seeds, two trays for hydrocarbon contaminated soils and the other two containing uncontaminated soils. Pot plates were used to cover the pot plants in order to avoid leaching. The trays were then kept in a green house under controlled conditions in an open area allowing light for photosynthesis. The seedlings were irrigated three times a week. The plants were allowed to grow for a period of six weeks before soil samples were taken for analysis.

3.3.3. Sample collection

Hydrocarbon contaminated and uncontaminated rhizosphere soil samples

Tap water was used to wet the soil before samples were taken. The wet soil was then loosened by mixing with a spatula. The different plant species (*E. coracana* and *B. pilosa*) were then slowly pulled out of the hydrocarbon contaminated and uncontaminated soil (not breaking the roots) and then shaken to remove the soil attached to the roots. Hydrocarbon contaminated and uncontaminated rhizosphere soil samples were collected in separate plastic bags. 10g of hydrocarbon contaminated and uncontaminated rhizosphere soil were homogenized in 90ml of sterile Ringer's solution separately, and the suspensions were shaken in a rotatory shaker incubator at 200rpm at 22°C for 30 min. The mixture was then allowed to settle separating the supernatant and the precipitate for 30 min. Thereafter; serial dilutions of 10^{-2}, 10^{-4} and 10^{-6} were made in sterile Ringer's solution.

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3.3.4. Inoculation of the Ecoplate (Biolog)

The procedure used in this study was similar to that reported by Garland and Mills (1991) and can be summarized as follows:

125µl aliquots of each dilution (10^{-2}, 10^{-4}, 10^{-6}) were inoculated in each of the Ecoplate wells, including sterile water as a control. Plates were incubated in the dark at 37°C in a rotatory incubator for 24, 48, 72 and 96 h. The utilization of the carbon sources resulted in colour formation from colourless to purple.

3.3.5. Total Plate counts

Serial dilutions (10^{-2}, 10^{-4} and 10^{-6}) of the hydrocarbon contaminated and uncontaminated rhizosphere soil samples were made using sterile Ringer's solution as diluent. 100µl of the hydrocarbon contaminated and uncontaminated rhizosphere soil samples were spread in duplicate on Nutrient agar plates supplemented with cyclohexamine and incubated at 37°C for 48h. The colonies were counted and expressed as cfu/ml.

3.4. Results and discussion

3.4.1. Total bacterial numbers

Table 3.1. Number of culturable microorganisms in the rhizosphere soil of *E. coracana* and *B. pilosa* growing in hydrocarbon contaminated and uncontaminated soils.

Soil type	Plant species	Plate count (cfu/g)
Contaminated	*Elusine coracana*	8.9×10^{7}
Contaminated	*Biddens pilosa*	3.7×10^{7}
Uncontaminated	*Elusine coracana*	1.9×10^{8}
Uncontaminated	*Biddens pilosa*	9.8×10^{7}

cfu = colony forming units

The highest number of bacteria was obtained in the rhizosphere of *E. coracana* grown in uncontaminated soil, followed by *B. pilosa* growing in uncontaminated soil, followed by *E. coracana* and *B. pilosa* growing in contaminated soil. The range of bacterial numbers varied between 3.7×10^{7} cfu/g and 1.9×10^{8}cfu/g (Table 3.1). The bacterial number in the contaminated rhizosphere soils was slightly lower than the

uncontaminated soil. It is however difficult to conclude that this was of any significance. According to our hypothesis, the diversity of the organisms rather than the total number will be more reliable indicator of any selective pressure exerted by the contamination.

3.4.2. Species diversity

Colour development in each well of the Biolog (Ecoplates) microtiter plates reflected the ability of the bacterial community to utilize specific substrates. The results indicated that incubation periods of 24 and 48h were not sufficient to complete colour development in the Ecoplate microtiter plate wells. The results indicated complete colour development after incubation periods of 72 and 96h and hence all plates were incubated for 96h during this study.

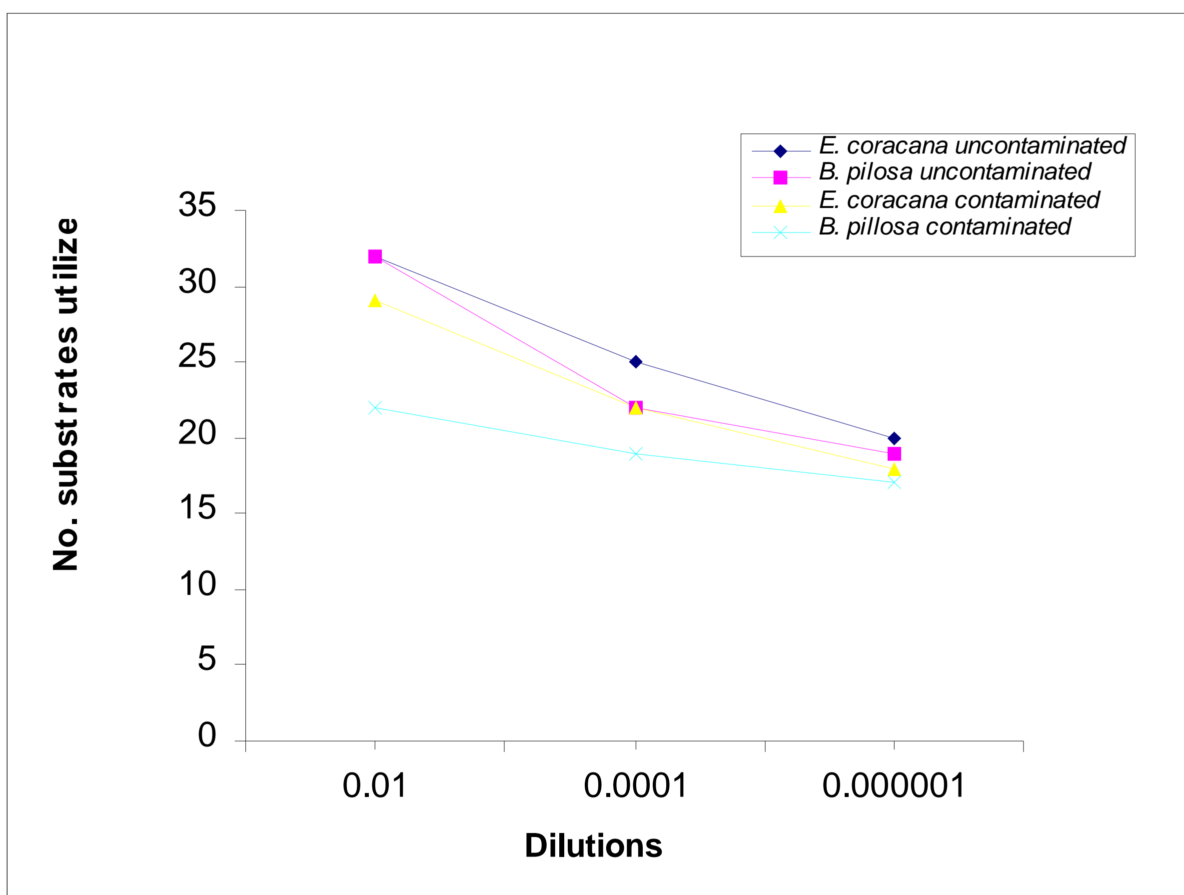

Figure 3.1. Substrate utilization for the different dilutions (10^{-2}, 10^{-4} and 10^{-6}) of hydrocarbon contaminated and uncontaminated rhizosphere soils of *Elusine coracana* and *Biddens pilosa* plants.

In the *Biddens pilosa* and *Elusine coracana* growing in the uncontaminated soil, all of the substrates (31) in the Ecoplates were utilized followed by *E. coracana* (22 substrates utilized) and *B. pilosa* (22 substrates utilized) in the contaminated soil. This indicated a higher microbial diversity in the uncontaminated rhizosphere soil samples compared to the contaminated rhizosphere samples. *B. pilosa* hydrocarbon contaminated rhizosphere soil had the lowest diversity as indicated by fewer substrates utilized (19 substrates). Upon dilution (10^{-2}, 10^{-4} and 10^{-6}) of the rhizosphere soil a decline in the number of Ecoplate substrates utilized was observed (Fig 3.1). This indicated an uneven distribution of the organisms with a loss of species upon dilution in all the rhizosphere samples (Fig 3.1).

The decline (gradient) with *B. pilosa* grown in contaminated soil was not as steep as the other three soils. This indicated that the microbial community remained constant upon dilution. This suggested that selection for species that could tolerate the contamination may have taken place. The organisms which survived will be those that can tolerate the stress and may therefore be the most effective in terms of bioremediation and this will need to be further investigated.

3.5. Conclusions

- An incubation period of 96h was optimal for the colour development in the microtiter plates.
- The total bacterial counts indicated a high number of bacteria in all the rhizosphere soils with the uncontaminated soil having a marginally higher number.
- The species diversity in the uncontaminated rhizosphere was high, but unevenly distributed.
- The species diversity in the rhizosphere of *B. pilosa* grown in contaminated soil was lower and evenly distributed indicating predominance of certain bacteria.

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Chapter 4

Identification of microorganisms on the rhizosphere of plants grown in Petroleum contaminated and uncontaminated soils

4.1. Abstract

Large areas of soil are polluted with recalcitrant organic substances that pose environmental problems due to their toxicity and tendency to disperse through wind and water erosion. Field trials have shown that remediation of Petroleum contaminated sites can be enhanced by cultivation of plants. To date, a great variety of grasses, legumes and fast growing trees rates have been applied during remediation of Petroleum contaminated soil. Grasses were the most promising plants since they harbour large numbers of bacteria on their highly branched root systems, some of which can be useful in cleaning up the environments. Thus, these plant species could be used during bioremediation of contaminated soil. Screening of bacteria isolated from the rhizosphere of *Cyperus esculentus*, *Elusine coracana*, *Biddens pilosa*, *Brantha serratia* grown in Total Petroleum Hydrocarbons (TPHs) and uncontaminated soil resulted in the collection of 12 bacterial isolates. Isolates were classified morphologically and identified using API. Biolog was also used for the identification of Gram-negative bacteria. Bacterial strains belonged to the genera *Brevibacillus*, *Staphylococcus*, *Brevundimonas*, *Acinetobacter*, *Sphinomonas*, *Pseudomonas*, *Vibrio*, *Flavobacterium*, *Chryseo*, *Micrococcus* and *Bacillus*.

Key words: Total Petroleum Hydrocarbons, Bacteria, *Cyperus esculentus*, *Brantha* serratia, *Elusine coracana*, Bioaugmentation, Environment, Bioremediation.

4.2. Introduction

The numerous classes and types of chemicals, which contaminate soil,

complicate the removal of many potentially toxic compounds from the environment. For example, many soils are contaminated with one or more metals, radioactive or inorganic compounds. Of these, the metals may include lead, zinc, cadmium, selenium, chromium, cobalt, copper, nickel and mercury; the radioactive compounds may be uranium, cesium or strontium; and the other inorganic compounds might include arsenic, sodium, nitrate, ammonia or phosphate (Glick, 2003; Molas *et al*., 2004).

A high level of metal accumulation in the soil and in ground and surface waters poses a threat to normal functions of plants and both directly and indirectly in humans (Videa *et al*., 2004). An increase in the level of the phytoavailability of necessary (Cu^{2+}, Zn^{2+}, Mn^{2+}) and nonessential (Cd^{2+}, Pb^{2+}, Hg^{2+}) metals is harmful (Piechalak *et al*., 2003). Many studies have been conducted in order to identify plant species capable of accumulating undesirable toxic compounds such as heavy metals. Reeves *et al*. (2000) compiled a list of plant species that hyperaccumulate Cd, Cr, Ni, Pb, Se and Zn.

Large areas of soil are polluted with recalcitrant organic substances that pose environmental problems due to their toxicity and tendency to disperse through wind and water erosion (Joner *et al*., 2004; Sabate *et al*., 2004). Oil spills pose a serious risk to the health of wetland systems throughout the world (Lin *et al*., 2002). Soil pollution by petroleum is a widespread problem. Diesel fuel, a common contaminant, has a high content of alkanes between C^{8} and C^{26}. Alkanes are pollutants comparatively easy to degrade, as alkenes between C^{10} and C^{25} are biodegradable (Tesar *et al*. 2002).

Diesel fuel, on entering the terrestrial environment, will spread and seep into the soil. The downward migration of diesel fuel through the soil profile is limited due to the physical properties of the soil. Under normal conditions, diesel fuel will be adsorbed in the organic rich surface soil, impeding downward migration (Gestel *et al*., 2003). This makes diesel fuel contaminated soil a likely candidate for phytoremediation as the contaminant is held in the surface soil and within the rooting zone of most plant species (Adam *et al*., 2002).

Excavated petroleum contaminated soil can be bioremediated by addition of nutrients (bioaugmentation), aeration and turning, or by combination of these practises. Also, the addition of organic matter to the hydrocarbon contaminated soil can be beneficial, as it is a source of co- substrate, nutrients and microorganisms, and ameliorates the structure and water-retaining capacity of the soil (Coosemans *et al*., 2003).

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous persistent environmental pollutants derived from natural and anthropogenic processes (Guerin, 1999). The marine environment as a reservoir receives a large amount of PAHs from oil spills, ship traffic, urban run off, wastewater and industrial discharge, as well as atmospheric deposition of vehicle exhaust and industrial stack emission (Vervaeke *et al*., 2003).

As PAHs enter into the marine environment, they bind tightly to suspended particles and accumulate in bottom sediments due to their low water solubility and hydrophobic properties. The PAHs in marine sediments being recalcitrant and persistent would have a strong tendency to be concentrated in marine food webs. Because of the known or suspected toxic, mutagenic and carcinogenic nature, PAHs would cause

health risks to human being (Ke *et al*., 2003).

Because Polyaromatic Hydrocarbons (PAHs) are genotoxic and carcinogenic, they represent a considerable environmental concern. As abiotic factors do not contribute significantly to the elimination of PAHs, much effort has been expended in examining the contribution of microorganisms to degrade PAHs. Low molecular weight PAHs are readily degraded. However, high molecular weights PAHs (four and more rings) are more persistent because of their low bioavailability and their strong adsorption onto the soil organic matter (Potin *et al*., 2004).

Ramsay *et al*. (2000) discovered that the indigenous microbial community containing alkane- and PAH- degrading bacteria could have a considerable potential to remedy the oil- contaminated mangrove sediments. Sotsky *et al*. (1994) also reported that most culturable soil bacteria (gram- positive and gram- negative) occurring at hydrocarbon polluted environments contain genes encoding oxygenases. These enzymes, including alkane hydroxylase (encoded by alkB), the prototype of non- haem, iron intergral membrane monooxygenases, are essential for monoterminal oxidation, which is the main pathway of alkane degradation.

One recently developed method of environmental clean up is called phytoremediation. This procedure may be defined as the use of plants to remove, destroy or sequester hazardous substances from the environment

(Glick *et al*., 2003). Field trials have shown that remediation of petroleum contaminated sites can be enhanced by cultivation of plants. To date, a great variety of grasses species, legumes and fast growing trees with high transpiration rates such as poplar, alder or willow have been applied for phytoremediation. These plants provide large surface areas for root soil contact due to their expansive root system. Roots provide ideal attachment sites for microbes and a food supply of exudates consisting of amino acids and organic acids, sugars, enzymes and complex carbohydrates ((Mehmannavaz *et al*., 2002; Tesar *et al*., 2002).

Jordahl *et al*. (1997) reported that the microbial numbers degrading benzene; toluene and xylene were five times more abundant in the rhizosphere of poplar trees than in the surrounding soil. A broad phylogenetic range of bacteria including species/ strains of *Achromobacte* r, *Acidovorax*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Mycobacteium*, *Norcardia*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas* and *Xanthomonas* have been identified in the breakdown of hydrocarbons (Tesar *et al*., 2002).

The advantages of phytoremediation are: (1) it is an aesthetically pleasing, solar- energy driven clean- up technology, (2) there is minimal environmental disruption and in situ treatment preserves topsoil, (3) it is most useful at sites with shallow, low levels of contamination, (4) it is useful for treating a broad range of environmental contamination and (5) it is less expensive (60- 80% or even less costly) than the conventional physico- chemical methods. On the other hand, phytoremediation has its limitations. It is a time consuming process, and it may take at least several growing seasons to clean up a site (Morikawa *et al*., 2003).

Actively growing roots release organic compounds into the rhizosphere such as sloughed off cells, excretions, lysates and exudates. These

compounds support growth of the microbial community in the rhizosphere and will result not only in an increased population density, but also in a community structure distinct from that in the bulk soil (Soderberg *et al*., 2004).

Biodegradation of complex hydrocarbons usually requires the cooperation of more than a single species. This is particularly true for pollutants that are made up of many different compounds such as crude oil or petroleum where complete mineralization to CO_2 and H_2O is desired. Individual microorganism can metabolise only a limited range of hydrocarbons substrate, so assemblage of mixed populations with overall broad enzymatic capacities are required to bring the rate and extent of petroleum biodegradation further. Microbial populations that consist of strains that belong to various genera have been detected in petroleum-contaminated soil or water. This strongly suggests that each strain or genera have their role in the hydrocarbon transformation processes (Ghazali *et al*., 2004).

Beneficial free-living soil bacteria are generally referred to as plant growth promoting rhizobacteria that are found in close association with the roots of many different plants. The high concentration of bacteria around the roots, i.e. in the rhizosphere, presumably occurs because of the presence of high levels of nutrients (especially small molecules such as amino acids, sugars and organics acids) that are exuded from the roots of most plants. The exudation can then be used to support bacterial growth and metabolism. Plant growth promoting bacteria can positively influence plant growth and development. Promotion of plant growth occurs when plant growth promoting bacteria prevent some of the deleterious effects of phytopathogenic organisms. These bacteria can directly promote plant growth by providing the plant with a compound that is synthesized by a bacterium or by facilitating the uptake of nutrients from the environment

by the plant (Glick *et al*., 2003).

The objective of this research was to isolate and purify bacterial cultures obtained from the rhizosphere of plants grown in total petroleum hydrocarbons (TPHs) contaminated and uncontaminated soil, to differentiate cultures morphologically using standard microbiological techniques and to identify these cultures.

4.3. Materials and methods

4.3.1. Study sites and sampling procedures

Two sites close to a refinery were studied: an excavation site, which has a history of total petroleum hydrocarbons (TPHs) contamination and an uncontaminated site that is free from petroleum hydrocarbons. Spades were used to loosen the plants and the plants were slowly pulled out of the ground (not breaking the roots). Plants were then shaken to remove soil attached to the roots. Hydrocarbon contaminated and uncontaminated rhizosphere soil samples were collected in separate marked plastic bags and stored at 4°C until used.

4.3.2. Sample preparation

Rhizosphere contaminated and uncontaminated soils were air dried and passed through a 2mm sieve. Soils were then homogenized by taking 10g of dry soils of each sample and adding to 90ml sterile ¼ strength Ringer's solution (Merck) in 250ml Erlenmeyer flasks and allowed to shake for 1h in a shaker incubator. After mixing serial dilutions were made and 100µl of the 10^{-4} was plated out on Tryptone soy agar plates (TSA) added with cycloheximide for determining culturable bacteria. Plates were incubated at 37°C for 48h.The bacterial colonies that developed on the plates, were randomly picked and purified by subculturing on fresh TSA agar plates

using the streak plate technique. The bacterial isolates were classified based on their morphology, Gram reaction and their biochemical reaction.

4.3.3. Catalase reaction

Cells from bacterial isolates were picked using wooden toothpick and put on cleaned microscope slides. One or two drops of 3% hydrogen peroxide were added on the slides. The formation of bubbles within 1 min was regarded as positive.

4.3.4. Determination of aerobic and anaerobic bacteria

Fermentative and oxidative bacteria were determined and procedures were similar to that of Hugh- Leifson (1953) and can be summarized as follows: colonies from Tryptone Soy Agar (TSA) plates were stab inoculated in sterile test tubes containing oxidative fermentative (OF) Base medium mixed with glucose using flamed and cooled needle. Two test tubes were use for each bacterial isolate: one tube containing OF Base medium mixed with glucose, isolate and sterile paraffin liquid on top of the medium (1cm deep) and the other tube containing OF Base medium mixed with glucose and isolate. Test tubes were incubated at 37 ^{o}C for 48h and colour change from green to yellow is positive. Fermentative bacteria resulted in colour changed from green to yellow in both test tubes whereas oxidative bacteria were resulted by colour changed from green to yellow in a test tube that contained no liquid paraffin.

4.3.5. Identification of bacterial species using API and Biolog

Pure isolates were subcultured on fresh agar for 48h. A loopful of cells from pure colonies was suspended in sterile test tubes containing 0.85% Nacl. The inoculation of the API strips was done according to manufacture's instructions (OMNIMED Company S.A.). The API used was

API 50CH, API 20NE and API 20E based on different characteristics of the bacterial isolates. Biolog was also used to identity Gram- negative bacteria. Procedure was done accorning to manufacture's instructions (Anatech Instuments).

4.4. Results and discussion

Table 4.1. No. of bacterial isolates obtained after 48h of incubation from different samples.

Samples	Bacterial counts (cfu/ml)	No. isolates obtained
A	$3.3x10^{+6}$	3
B	$3.05x10^{+6}$	3
C	$3.10x10^{+6}$	2
D	$1.97x10^{+6}$	2
E	$1.35x10^{+6}$	2
F	$1.48x10^{+6}$	2
G	$1.80x10^{+6}$	2
H	$2.68x10^{+6}$	2

Cfu = colony forming unit.

A=*Cyperus esculentus* contaminated; B=*Elusine coracana* contaminated; C=*Biddens pilosa* contaminated; D=*Biddens pilosa* uncontaminated; E=*Cyperus esculentus* uncontaminated; F=*Brantha serratia* uncontaminated; G=Contaminated without plants and H=Uncontaminated without plants.

4.2. Bacterial morphology, arrangement and catalase reaction

Plant name	Isolate	Gram staining	Morphology	Arrangement	Catalase reaction
1. *Cyperus esculentus* contaminated	A1	+	Rod	Cluster	+
	A2	+	Cocci	Chain	+
	A3	+	Rod	Chain	+
2. *Cyperus esculentus* uncontaminated	E1	_	Rod	Cluster	+
	E2	_	Cocci	Cluster	_
3. *Elusine coracana* contaminated	B1	_	Rod	Diplo	+
	B2	_	Rod	Diplo	+
4. *Biddens pilosa* contaminated	C1	_	Rod	Diplo	+
	C2	_	Rod	Chain	+
5. *Biddens pilosa* uncontaminated	D1	_	Rod	Chain	+
	D2	_	Rod	Cluster	_
6. *Brantha serratia* contaminated	F1	_	Rod	Cluster	+
	F2	+	Rod	Chain	+
7. Contaminated without plants	G1	+	Cocci	Tetra	_
	G2	+	Cocci	Cluster	+
8. Uncontaminated without plants	H1	_	Rod	Cluster	+
	H2	+	Cocci	Diplo	+

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4.3. Determination of oxidative and facultative bacteria

Plant name	Isolate	Fermentative	Oxidative
1. *Cyperus esculentus* contaminated	A1	+	_
	A2	Nc	Nc
	A3	_	+
2. *Cyperus esculentus* uncontaminated	E1	+	–
	E2	+	_
3. *Elusine coracana* contaminated	B1	_	+
	B2	_	+
4. *Biddens pilosa* contaminated	C1	+	_
	C2	+	_
5. *Biddens pilosa* uncontaminated	D1	Nc	Nc
	D2	_	+
6. *Brantha serratia* contaminated	F1	_	+
	F2	_	+
7. Contaminated without plants	G1	_	+
	G2	+	_
8. Uncontaminated without plants	H1	_	+
	H2	Nc	Nc

Nc =No colour formed

Sometimes when the organisms do not utilize glucose, there will be no colour change from green to yellow. If a greenish colour forms in the tube exposed to air, it may indicate proteins utilization instead of glucose.

4.4. Identification of bacteria isolated from the rhizosphere of plants grown in Total Petroleum Hydrocarbons (TPHs) contaminated and uncontaminated soil.

Plant name	Isolate	Identification
1. *Cyperus esculentus* contaminated	A1	*Brevibacillus brevis*
	A2	*Staphylococcus epidermidis*
	A3	*Brevundimonas vesicularis*
2. *Cyperus esculentus* uncontaminated	E1	*Acinobacter calcoaceticus*
	E2	*Sphinomonas parapaucimobilis*
3. *Elusine coracana* contaminated	B1	Acinobacter calcoaceticus
	B2	*Pseudomonas putida*
	B3	*Staphylococcus epidermidis*
4. *Biddens pilosa* contaminated	C1	*Vibrio vulnificus*
	C2	*Pseudomonas spinosa*
5. *Biddens pilosa* uncontaminated	D1	*Flavobacterium ferrigineus*
	D2	*Pseudomonas putida*
6. *Brantha serratia* contaminated	F1	Chryseo indologenes
	F2	*Brevibacillus brevis*
7. contaminated soil without plants	G1	*Micrococcus spp*
	G2	*Bacillus stearothermophilus*
8. Uncontaminated soil without plants	H1	*Pseudomonas putida*
	H2	*Staphylococcus epidermidis*

The bacterial isolates obtained from the different rhizosphere of contaminated and uncontaminated soils were identified to be of the following genera: *Bacillus*, *Brevibacillis*, *Pseudomonas*, *Acinobacter*, *Brevundimonas*, *Staphylococcus*, *Sphinomonas*, *Vibrio*, *Flavobacterium*, *Chryseo* and *Micrococcus* (Table 4.4.). The predominant forms were rod shaped bacteria and a few *cocci* (Table 4.2.). Most of the *Bacilli* were gram negative, catalase positive and oxidative (Table 4.2. and 4.3.). The total viable count ranged from 1.35×10^{-6} to 3.3×10^{-6} within 48h of incubation.

The dominant bacteria in the rhizosphere of *Cyperus esculentus* grown in contaminated soil were *Brevibacillus brevis*, *Brevundimonsa vescularis* and

Staphylococcus epidermidis (Table 4.4.). These dominant bacteria were different than the dominant bacteria in the rhizosphere of *C. esculentus* grown in ucontaminated soil i.e. *Acinobacter calcoaceticus* and *Sphinomonas parapaucimobilis* (Table 4.4.). This indicates a shift in the microbial population, as would be expected.

Acinobacter calcoaceticus, *P. putida* and *S. epidermidis* were the dominant bacteria isolated from the rhizosphere of *E. coracana* grown in contaminated soil. However, this plant was not found in uncontaminated soil.

The dominant bacteria in the rhizosphere of *B. pilosa* grown in contaminated soil were *Vibrio vulnificus* and *Pseudomonas spinosa*. These species were different from that isolated from the rhizosphere of *B. pilosa* grown in uncontaminated soil i.e. *Flavobacterium ferrigineus* and *P. putida* (Table 4.4.). This also indicated a shift in microbial population.

Rhizosphere of *Brantha serratia* grown in contaminated soil has been found to consist of dominant bacteria, which were *B. brevis* and *Chryseo indologenes* (Table 4.4.). Finally *Micrococcus spp*, *Bacillus stearothermophilus*, *Pseudomonas putida* and *Staphylococcus epidermidis* were the dominant bacteria isolated from contaminated and uncontaminated soils with no plants (Table 4.4.).

E. coracana and *B. serratia* were not found in uncontaminated soil. This may be due of unfavourable conditions that inhibited the growth of these plants. External environmental factors and insufficient supply of nutrients do sometimes disturb the growth of plants.

Studies of plant species for rhizoremediation showed that various grass varieties and leguminous plants were suitable for biodegradation (Kuiper

et al., 2003). In this study, *Cyperus esculentus*, *Elusine coracana* and *Brantha serratia* (grasses) supported the largest variety of bacteria, which supports the finding of Kuiper *et al*. (2003).

This probably is due to their ability to harbour large numbers of bacteria on their highly branched root system. Successful rhizoremediation also depends on factors such as primary and secondary metabolism, colonization, survival and ecological interactions with other organisms. Hence, plant roots were suggested as a substitute for the tilling of soil to incorporate additives and to improve aeration as a method of remediation (Kuiper *et al*., 2003).

The use of plants in combination with microbes has the advantage of causing an increase in microbial numbers and metabolic activity in the rhizosphere. Rhizoremediation can also result in an improvement of the physical and chemical properties of contaminated soil, and an increase in contact between the microbes associated with the roots and the contaminants in soil (Aprill *et al*., 1990).

This stimulatory rhizosphere effect has been recognized for many years and was describe for the first time by Hiltner (1904), who defined the rhizosphere as the zone soil in which microbes are influenced by the root system. In turn, rhizosphere organisms also have a large impact on plants, because many microbes isolated from the rhizosphere are described to have root growth stimulating or growth inhibiting properties (Kuiper *et al*., 2003).

The exudation of nutrients by plant roots creates a nutrient-rich environment in which microbial activity is stimulated. Plant root exudates contain sugar, organic acid and amino acids as main components. In addition, the mucigel secreted by root cells, lost root cap cells, the

starvation of root cells or the decay of complete roots provide nutrients (Reilley *et al*., 1996).

The composition of the microbial population in the rhizosphere depends on the composition of the root exudates as well as on the plant species, root type, plant age, soil type and history of soil. It is known that gram-negative rods such as *Pseudomonas spp* dominate the rhizosphere (Kuiper *et al*., 2003).

4.5. Conclusions

Grasses such as *Cyperus esculentus*, *Brantha serratia* and *Elusine coracana* are the most promising plants species since they harbour large numbers of bacteria on their highly branched root systems, some which can be useful in cleaning up the environments such as soils contaminated by Polyaromatic Hydrocarbons (PAHs). Thus, these plant species could be used during bioremediation of PAH-contaminated soils.

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Chapter 5

PAH degrading potential of bacteria isolated from the rhizosphere of plants grown in polluted soil and in polluted soil without plants.

5.1. Abstract

Polycyclic Hydrocarbons (PAHs) are widely distributed in the environment. Many PAHs are highly toxic, mutagenic and carcinogenic to microorganisms as well as to humans and animals. Although various physicochemical methods have been used to remove these compounds from the environment, they have many limitations. A number of microorganisms isolated from the rhizosphere of plants were capable of the biodegradation of Polycyclic aromatic Hydrocarbons. In this study, identified bacteria isolated from the rhizosphere of plants grown in contaminated soil at a refinery in South Africa were inoculated at different concentrations (1%, 3% and 5%) of naphthalene and acenaphthene based medium and incubated for 48h at 37 ^{o}C. Results showed that *Brevibacillus brevis*, *Brevundimonas versicularis*, *Vibrio vulnificus*, *Chryseo indologenes*, *Micrococcus spp*, *Bacillus stearothermophilus*, *Pseudomonas putida* and *Pseudomonas spinosa* were capable of growth in all naphthalene concentrations. However, *Micrococcus spp* and *Pseudononas spinosa* showed limited growth in acenaphthene based medium. It was concluded that *B. brevis, B. versicularis*, *V. vulnificus*, *C. indologenes*, *Micrococcus spp*, *B. stearothermophilus*, *P. putida* and *P. spinosa* were considered potential degraders of naphthalene because they could use naphthalene as carbon source.

Key words: Xenobiotic, Polycyclic aromatic Hydrocarbons, Toxic, Mutagenic, Carcinogenic, Bioremediation, Biodegradation.

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5.2. Introduction

The extensive use of petroleum products leads to the contamination of almost all components of the environment (Chaillan *et al*., 2004). Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants found in soil at wood preservation plants and gasworks (Aitken *et al*., 1997). Because they are genotoxic and carcinogenic, they represent a considerable environmental concern (Potin *et al*., 2004). As abiotic factors do not contribute significantly to the elimination of PAHs with more than three rings from soil, much effort has been expended in examining the contribution of microorganisms to degrade PAHs. Low molecular weight PAHs are readily degraded. However, high molecular weights PAHs (four and more rings) are more persistent, in part because of their low bioavailability, due to their strong adsorption onto the soil organic matter. Soil adsorption is especially strong in long term- contaminated soils, which is often the case of soils in the vicinity of gas manufacturing plants (Potin *et al*., 2004).

Polycyclic aromatic hydrocarbons (PAHs) are chemical compounds that persist in the environment and thus cause pollution worldwide (Chaineau *et al*., 1997). Their persistence in the environment is due to their low water solubility. Generally, PAH solubility decreases and hydrophobicity increases with an increase in number of fused benzene rings. In addition, volatility decreases with an increasing number of fused rings (Juhasz *et al*., 2000; Woo *et al*., 2004).

The major source of PAHs is from the combustion of organic material such

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as coal, tar wood and rubber (Reilley *et al*., 1996). PAHs are formed naturally during thermal geologic production and during burning of vegetation in forest and bush fire (Juhasz *et* al., 2000).

The release of PAHs into the environment is widespread since these compounds are ubiquitous products of incomplete combustion. PAHs have been detected in a wide variety of environmental samples including air, soil, sediments, water, oils, tars and foodstuffs. Industrial activities such as possessing, combustion and disposal of fossil fuel, are usually associated with the presence of PAHs at highly contaminated sites (Woo *et al*., 2004). PAHs contamination on industrial sites is commonly associated with spills and leaks from storage tanks and with the conveyance, processing, use and disposal of these oil products (Juhasz *et al*., 2000).

Increasing petroleum exploration refining and other allied industrial activities in the Niger Delta have led to the wide scale contamination of most of its creek, swamps, rivers and streams with hydrocarbons and dispersant products. The contamination of these habitats constitutes public health and socio economic hazards. The xenobiotics discharge may also pose serious aquatic toxicity problems. Xenobiotics may affect the microorganisms, genetic machinery and population. The pollutant may inhibit some microbial communities that are important in some biogeochemical cycles of that ecosystem and this affects the productivity of such ecosystem (Okerentugba *et al*., 2003).

Most people are exposed to PAHs when they breathe smoke, auto emissions or industrial exhausts. Most exhausts contain many different PAH compounds. People with highest exposures are smokers, people who live or work with smokers, roofers, road builders and people who live near major highways or industrial sources. Charcoal-broiled food, especially meats, are a source of some PAH exposure. Shellfish living in contaminated water may be another major source of exposure. PAH may be in ground water near disposal sites where the construction wastes or ash are buried. Drinking this water may expose people. Vegetables do not take up significant amounts of PAHs that are in soil. PAHs can also be absorbed through skin. Exposure can come from handling contaminated soil or bathing in contaminated water. Low levels of these chemicals may be absorbed when a person uses medicated skin cream or shampoo containing PAHs (http//www.dhfs.state.wi.us/eh/chemFS/fs/PAH.htm).

Several PAHs are known to be toxic, mutagenic and carcinogenic. Remediation of contaminated sites with these compounds is of great environmental concern (Woo *et al*., 2004). Exposure to PAHs can lead to a variety of chronic or acute effects in humans and animals including various forms of cancer and birth effects in newborn (Reilley *et al.,* 1996). Long term exposure to Benzo[a]pyrene in humans has resulted in skin rashes, sensitivity sunlight, eye irritation and cataracts. BaP has been found to cause reproductive defects in animals and humans. BaP can result in decrease fertility and reduce incidence of pregnancy and can damage sperms and reproductive organs in males. This PAH also damages organ systems such as lungs, liver, skin and kidneys when expose to it (http//www.checnet.org/healthehouse/chemicals/chemicals-detail.asp?Main_ID=395).

The estimated costs for the clean up of contaminated sites with

conventional techniques such as incineration and landfilling are enormous. In the next decades, billions of dollars will be spent to clean up all sites polluted with Polyaromatic hydrocarbons. In the US alone, restoration of all contaminated sites will cost approximately 1.7 trillion dollars. In addition the conventional techniques are not always sufficient. Incineration can results in air pollution, leaches from landfills in the form of water and gases can reach the ground water and drinking water wells, whereas excavation of soil can lead to the generation of toxic air emission (Gestel *et al*., 2003).

The search for alternative methods to restore polluted sites in a less expensive, less labor intensive, safe and environmentally friendly way is required. Such an alternative method is bioremediation, which is defined as the action of microbes or other biological systems to degrade environmental pollutants (Kuiper *et al*., 2003).

Bioremediation has been studied extensively in the last two decades as a means of removing PAHs, especially from contaminated soils (Lee *et al*., 2003). Soil pollution by petroleum products is a wide spread problem. Bioremediation techniques have been developed and improved to clean up soils polluted with hazardous chemicals. Excavated petroleum contaminated soil can be bioremediated by addition of nutrients (biostimulation), addition of microbial inocula (bioaugmentation), aeration and turning, or by combination of these practices. Also, the addition of organic matter to the hydrocarbon contaminated soil can be beneficial, as it is a source of substrates, nutrients and microorganisms and ameliorates the structure and water retaining capacity of the soil (Gestel *et al*., 2003).

Bioremediation can be applied in situ without the removal and transport of polluted soil and without the disturbance of the soil matrix. Another advantage is that bacterial degradation usually results in complete

mineralization of the pollutants (Kuiper *et al*., 2003).

When a site gets polluted, the composition of the indigenous microbial population in the soil and ground water will adapt to new situation. Bacteria able to use the pollutant substrates as a nutritional source will be able to proliferate and may become dominant. The natural, non-engineered process of degradation of xenobiotics by the indigenous microbial population is referred to as natural attenuation, and is regarded as the simplest form of bioremediation (Kuiper *et al*., 2003).

Microbial communities exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes resulting in increased proportions of hydrocarbon- degrading bacteria and bacterial plasmid encoding hydrocarbon catabolic genes. Adapted microbial communities have higher proportions of hydrocarbon degraders that can respond to the presence of hydrocarbon pollutants (Okerentugba *et al*., 2003)

An ability to isolate high numbers of certain oil degrading microorganisms from an environment is commonly taken as evidence that those microorganisms are the active degraders of that environment. A number of well- known microorganisms are responsible for the biodegradation of oil hydrocarbons. Bacteria have evolved regulatory systems that ensure the synthesis of enzymes so that the initial attack of these compounds is induced only when required. Thus, for an organism with the genetic information for utilizing benzene as carbon source, the enzyme for degrading benzene is induced when benzene reaches the bacterial environment. Some of these organisms have evolved an additional and highly effective system for responding to a variety of potential growth substrate (Woo *et al*., 2004).

Numerous genera of fungi, bacteria and algae have been observed to

oxidize PAHs (Dean- Ross *et al*., 2001). While there is a great diversity of microorganisms capable of degrading the low molecular weight PAH, such as naphthalene, acenaphthene and phenanthrene, relatively few genera have been observed to degrade the high molecular weight PAHs such as BaP (Juhasz *et al*., 2000).

Biodegradation of complex hydrocarbon usually requires the cooperation of more that a single species. This is particularly true in pollutants that are made up of many different compounds such as crude oil or petroleum and complete mineralization to CO_2 and H_2O is desired. Individual microorganism can metabolize only a limited range of hydrocarbon substrates, so assemblages of mixed populations with overall broad enzymatic capacities are required to bring the rate and extent of petroleum biodegradation further (Ghazali *et al*., 2004).

Microbial populations that consist of strains that belong to various genera have been detected in petroleum soil or water. This strongly suggests that each strain or genera have their roles in the hydrocarbon transformation processes (Ghazali *et al*., 2004).

A number of physical, chemical, biological or environmental factors may influence the rate and extent of PAH degradation. PAHs degradation may not occur due to the lack of essential nutrients or growth substrates, suboptimum temperature, oxygen availability or pH. PAHs may not be accessible due to its low water solubility (K_{ow}) or it may be at a concentration where it is toxic to bacteria, fungi or algae. Insufficient microorganisms may also limit the degradation of PAHs (Juhasz *et al*., 2000).

Plant enzymes establish the degradation of pollutants during phytoremediation, whereas, during normal attenuation or

bioaugmentation, the indigenous microbial population performs the degradation. In many of these studies, an important contribution to the degradation of pollutants is ascribed to microbes present in the rhizosphere of plants used during phytoremediation or of plants, which are emerging as natural vegetation on a contaminated site (Burken *et al*., 1996).

This contribution of the rhizomicrobial population is referred to as rhizoremediation. In some cases, rhizosphere microbes are even the main contributors to the degradation process. A plant can be considered to be a solar driven biological pump and treatment system, attracting water with its root system, accumulating water soluble pollutants in the rhizosphere, and concluding with the degradation or translocation of the pollutants (Chaney *et al*., 1997).

The first studies toward degradation of compounds in the rhizosphere mainly focused on the degradation of herbicides and pesticides. Today, many studies deal with degradation of hazardous organic compounds such as TCE, PAHs and PCBs. Studies of the most suitable plant species for rhizoremediation showed that various grass varieties and leguminous plants such as *Alfalfa* are suitable. This probably is due to their ability to harbor large numbers of bacteria on their highly branched root systems (Kuiper *et al*., 2003).

Success also depends on the factors such as primary and secondary metabolisms, and establishment, survival and ecological interactions with other microorganisms. The use of plants in combination with microbes has the advantage of causing an increase in microbial population numbers and metabolic activity in the rhizosphere. It also can establish an improvement of the physical and chemical properties of contaminated soil, and an increase in contact between the microbes associated with the

roots and the contaminants in soils (Kuiper *et al*., 2003).

The main reason for the improved degradation of pollutants in the rhizosphere is presumably the increase in the number and metabolic activity of microbes. The exudation of nutrients by plant roots creates a nutrient- rich environment in which microbial activity is stimulated. Plant root exudates contain sugar, organic acids and amino acids as main components. The composition of the microbial population in the rhizosphere depends on the composition of the root exudates as well as on the plant species, root type, plant age, soil type and history of soil (Alkorta *et al*., 2001). It is known that the rhizosphere is dominated by gram negatives rods such as *Pseudomonas spp* (Reilley *et al*., 1996).

The objective of this study is to evaluate the potential of bacteria isolated from the rhizosphere of plants grown in polluted soil for their ability to bioremediate PAHs.

5.3. Materials and Methods

5.3.1. Chemicals

Naphthalene (99%), Acenaphthene (99%) and Chloroform (99.8%) were purchased from Sigma Aldrich S.A. Bacterial media were purchased from Merck S.A.

5.3.2. PAH- degrading bacteria

Bacteria were isolated from the rhizosphere of plants grown in polluted sites located at a refinery in South Africa. According to the identification as described in chapter 4, the bacteria belonged to the genera of

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Brevibacillus, *Staphylococcus*, *Acinetobacter*, *Pseudomonas*, *Brevundimonas*, *Sphinomonas*, *Flavobacterium, Vibrio, Micrococcus*, *Bacillus* and *Chryseo*.

5.3.3. Determination of bacterial growth at different concentrations in PAH-based agar

Bacterial growth and degradation studies were performed in bacteriological agar containing added PAHs. Chloroform was added to naphthalene and acenaphthene to dissolve the compounds. Chloroform was allowed to evaporate overnight. The soluble compounds were sterilized by filtering through 0.20µ filter papers. Concentrations of 1%, 3%, and 5% of naphthalene and acenaphthene concentrations were made in sterile conical flasks. 500ml of bacteriological agar was prepared in 1000ml bottle and autoclaved at 121 ^{O}C for 15 min. The agar was allowed to cool too 45 ^{O}C. The different concentrations of naphthalene and acenaphthene were added to 1000ml bottles containing agar. The bottles were swirled gently to avoid air bubbles until the PAHs are totally mixed with the agar. The agar containing of naphthalene and acenaphthene was poured into Petri dishes in duplicates and allowed to set. After 30 min, 1000µl of the selected bacterial suspensions were spread onto the surface of PAH-based agar and incubated at 37 ^{O}C for 48h. After incubation, growth of bacteria was determined at each concentration and the growth was differentiated in terms of excellent, limited and no growth.

5.4. Results and discussion

Table 5.1. PAH-degrading potential of bacteria isolated from the rhizosphere of plants grown in polluted soil

Polyaromatic hydrocarbons

	Naphthalene concentration (%)			Acenaphthene concentration (%)		
Bacteria	1	3	5	1	3	5
Brevibacillus brevis	+++	+++	+++	+++	+++	+++
Staphylococcus epidermidis	+++	++	++	++	++	+++
Brevundimonas versicularis	+++	+++	+++	+++	+++	+++
Acinetobacter calcoaceticus	++	++	++	+++	++	++
Vibrio vulnificus	+++	+++	+++	+++	+++	+++
Chryseo indologenes	+++	+++	+++	+++	+++	+++
Micrococcus spp	+++	+++	+++	+++	++	++
Bacillus stearothermophilus	+++	+++	+++	+++	+++	+++
Pseudomonas putida	+++	+++	+++	+++	+++	+++
Pseudomonas spinosa	+++	+++	+++	+++	_	_

+++ = excellent growth

++ = limited growth

_ = no growth

Ten bacterial species isolated from the rhizosphere of plants grown in contaminated soil were tested for their ability to use naphthalene and acenaphthene as their carbon source at different concentrations i.e. 1%, 3% and 5%.

An incubation period of 48h to 168h resulted in the growth of all isolates in naphthalene and acenaphthene based media at various concentrations i.e. 1%, 3% and 5%. *B. brevis*, *B. versicularis*, *V. vulnificus*, *C. indologenes*, *Micrococcus spp*, *B. stearothermophilus*, *P. putida and P. spinosa* showed excellent growth in all concentrations in naphthalene based medium (Table 5.1.).

These bacterial isolates were considered potential degraders of naphthalene because they could use naphthalene as carbon source. These results were similar to that of Annweiller *et al*. (2000) who found that *Bacillus thermoleovorans* degraded naphthalene at 60 °C. They isolated *B. thermoleovorans* from contaminated compost and inoculated it in a liquid mineral salt medium containing naphthalene as the sole carbon source.

Ghazali *et al*. (2004) speculated that *Bacillus* species are more tolerant to high concentrations of hydrocarbons in soil due to their resistant endospores. It was also evident that isolates belonging to *Bacillus spp* could be more effective in cleaning up oil spills and Polyaromatic Hydrocarbons (PAHs) pollution (Ghazali *et al*., 2004). In this study three *Bacillus spp* were isolated and capable of growing on naphthalene and acenaphthene.

S. epidermidis showed excellent growth in 1% naphthalene. However, the growth became limited with an increase in naphthalene concentrations (3% and 5%). This means that the metabolism of this bacterium was limited with an increase in naphthalene. *A. calcoaceticus* showed limited growth in all concentrations of naphthalene (Table 5.1.).

It was therefore concluded that *Staphylococcus epidermidis* and *Acinetobacter calcoaceticus* had limited potential as PAH- degraders because of their limited growth in the presence of naphthalene.

In acenaphthene- based medium, *B. brevis*, *B. versicularis*, *V. vulnificus*, *C. indologenes*, *B. stearothermophilus*, and *P. putida* showed excellent growth at all the concentrations (Table 5.1.).

Micrococcus spp and *Pseudomonas spinosa* showed limited growth in acenaphthene. Acenaphthene has a higher molecular weight compared to

naphthalene. This could be reason why these isolates showed limited growth. At 1% of acenaphthene, *Micrococcus spp* showed excellent growth, but an increase in concentrations (3% and 5%) limited growth.

A. calcoaceticus grown in 1% acenaphthene resulted in excellent growth but high concentrations resulted in limited growth (Table 5.1.).

S. epidermidis showed limited growth at concentrations of 1% and 3% but excellent growth at 5%.

P. spinosa did not grow at the concentrations of 3% and 5% but excellent growth was observed at 1% of acenaphthene.

The enzymes present in *Micrococcus spp* and *A. calcoaceticus* responsible for breaking down acenaphthene were active at the 1% concentration, but deactivated at 3% and 5% concentrations causing the growth to be limited (Table 5.1.). The limited growth of *S. epidermidis* at 1% and 3% might be because enzymes responsible for the induction of the degradation pathway were not activated at lower concentrations, but activated at 5% acenaphthene (Table 5.1.).

To conserve energy for vital- growth related processes, the enzymes needed by bacteria to degrade a particular compound are not normally synthesized unless the compound is present in the medium (Aitken *et al*., 1997).

The bacterial PAH catabolic genes are located on the large plasmids, together with the regulatory genes and in some cases, with genes responsible for chemotaxis toward the PAH. The presence of these catabolic plasmids is probably the cause of the adaptation of the indigenous microbes toward PAH pollutants, because they are refereed as

self-transmissible and horizontal transfer of naphthalene catabolic genes (Kuiper *et al*., 2003).

During the naphthalene metabolic pathway, an enzyme naphthalene oxygenase is synthesized to induce the degradation process by converting naphthalene to *cis*-1, 2-dihydro-1, 2-dihydroxy naphthalene while an enzyme 1, 2-dihydroxynaphthalene oxygenase will further this process by converting 1, 2-dihydroxynaphthalene to 2-hydroxychromene 2-carboxylic acid. This intermediate (2-hydroxychromene 2-carboxylic acid) will then be converted to 2'- hydroxybenzalpyruvate by an enzyme known as 2'hydroxybenzalpyruvate aldolase and Salicyladehyde dehydrogenase will finish the process by converting salicyladehyde + pyruvate to salicylate which is the end product. This biodegradation pathway in bacteria is normally induced by the presence of either the parent compounds or one of the pathway intermediates (Connors *et al*., 1980).

Aitken *et al*. (1997) speculated that if we are to learn how to control the biodegradation of compounds, we clearly must understand how the relevant enzymes are turned on. This process is particularly important for those compounds that do not serve as the growth substrates for bacteria, since they are not likely to be able to induce their own degradation. In naphthalene metabolism, the complete pathway for conversion of naphthalene to simple, common metabolic end products is inducible by the intermediate salicylate in the bacteria. In other words, the enzymes required for naphthalene metabolism are elevated to high levels in naphthalene-degrading bacteria when salicylate is introduced into the medium.

A control was set up with all bacterial isolates inoculated in bacteriological agar without PAHs (naphthalene and acenaphthene), and

results indicated no growth in any of the plates. This means that all the bacterial isolates used naphthalene and acenaphthene as carbon source.

Amongst all these isolates tested for their potential in utilizing PAHs, *B. brevis*, *B. versicularis*, *V. vulnificus*, *C. indologenes*, *B. stearothermophilus*, and *P. putida* were the most promising, since none of them were affected by any change in concentration either in naphthalene or acenaphthene.

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Chapter 6

General discussion

Many sites are contaminated with toxic compounds such as Polycyclic aromatic Hydrocarbons (PAHs) due to human activities. PAHs are persistent in soil because of their low water solubility. Because PAHs are toxic, mutagenic and carcinogenic to humans and animals, their elimination is of paramount importance. Different biochemical procedures such as incineration and landfilling have been used extensively in the removal of PAHs from contaminated soil. These procedures are expensive,

time consuming and require disposable sites. Therefore, an alternative method that is less expensive and does not require disposable sites is required. Such method is called rhizoremediation, which is the use of plants in association with microorganisms to utilize PAHs. Although rhizoremediation is time consuming, it is more effective than the biochemical procedures. Plant roots release compounds such as amino acids, sugar, enzymes, aliphatics and aromatics to encourage the growth of rhizosphere microbial communities. Also, the presence of O_2 on the rhizosphere can stimulate the growth of rhizosphere microorganisms. In this study, *Brevibacillus brevis*, *Brevindimonas versicularis*, *Staphylococcus spidermidis*, *Acinetobacter calcoaceticus*, *Vibrio vulnificus*, *Chryseo indologenes*, *Micrococcus spp*, *Bacillus stearothermophilus*, *Pseudomonas putida* and *Pseudomonas spinosa* were isolated from the rhizosphere of *Elusine coracana*, *Cyperus esculentus*, *Brantha serratia* and *Biddens pilosa* grown in polluted site at a refinery in S.A. These bacteria were tested for their potential in breaking down naphthalene and acenaphthene at different concentrations (1%, 3% and 5%). It was shown that *B. brevis*, *B. versicularis*, *V. vulnificus*, *C. indologenes*, *B. stearothermophilus* and *P. putida* could not be affected by any change in concentration either in naphthalene and acenaphthene. However, *Micrococcus spp* and *P. spinosa* showed limited growth in acenaphthene concentrations. Same applied to *S. epidermidis*, an increase in naphthalene concentrations resulted in a decrease in the growth.

In order to learn how to control the biodegradation of PAHs, we must understand how the relevant enzymes are turned on. This process is important for those compounds that do not serve as growth substrates for bacteria, since they are not likely to be able to induce their own

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degradation. For example, in naphthalene metabolism, the complete pathway for the conversion of naphthalene to simple, common end products is inducible by the intermediate salicylate in the bacteria. In order words, the enzymes required for naphthalene metabolism are elevated to high levels in naphthalene- degrading bacteria when salicylate is introduced into the medium.

Summary

Numerous classes and types of chemicals, which contaminate soil, complicate the removal of many toxic compounds from the environment. For example, many soils are contaminated with one or more metals, radioactive and inorganic compounds. Large areas are polluted with recalcitrant organic substances that pose environmental problems due to their toxicity and tendency to disperse through wind and water errosion. Polycyclic aromatic Hydrocarbons (PAHs) are ubiquitous pollutants found in soil at wood preservation plants and gasworks. PAHs are chemical compounds that persist in the environment and thus cause pollution worldwide. Their persistence in the environment is due to their low water solubility. The major source of PAHs is from the combustion of organic material such as coal, tar, wood and rubber. PAHs have been detected in a wide variety of environmental samples including air, soil, sediments, water, oils, tars and foodstuff. Most people are exposed to PAHs when they breathe smoke, autoemissions or industrial fumes. Because PAHs are toxic, mutagenic and carcinogenic to humans and animals, their elimination from the environment is of paramount importance. The estimated costs for the clean up of PAHs contaminated sites with conventional techniques such as incineration and landfilling are enormous. The search for alternative methods to restore polluted sites in a less expensive, less labour intensive, safe and environmentally friendly way is required. Such an alternative method is rhizoremediation, which is defined as the use of plants in association with microorganisms to degrade environmental pollutants such as PAHs. Microbial communities exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes resulting in an increased proportion of hydrocarbon degrading bacteria and bacteria plasmid encoding hydrocarbon catabolic genes. Adapted microbial communities have higher proportions of hydrocarbon degraders that can respond to the presence of hydrocarbon

pollutants. The aim of this study was to identify bacteria isolated from the rhizosphere of *Elusine coracana*, *Biddens pilosa*, *Brantha serratia* and *Cyperus esculentus* grown in polluted and unpolluted soil and to evaluate the potential of bacteria isolated from the rhizosphere of these plants grown in polluted soil for their ability to bioremediate Polycyclic aromatic Hydrocarbons (PAHs).

Different concentrations (1%, 3% and 5%) of naphthalene and acenaphthene (PAHs) were made and added to 500ml Bacteriological agar.1000µl of bacterial suspensions were spread onto the surface of naphthalene and acenaphthene- based agar plates and incubated for 48h at 37 ^{o}C. Results showed that *Brevibacillus brevis*, *Brevindimonas versicularis*, *Vibrio vulnificus*, *Chryseo indologenes*, *Micrococcus spp*, *Bacillus stearothermophilus*, *Pseudomonas putida* and *Pseudomonas spinosa* showed excellent growth in all concentrations in naphthalene based agar medium. However, *Micrococcus spp* and *Pseudomonas spinosa* showed limited growth in acenaphthene based agar medium. Amongst all these bacteria tested for their potential in utilizing PAHs, *B. brevis*, *V. vulnificus*, C. *indologenes*, *B. stearothermophilus*, and *P. putida* were the most promising for biodegradation of PAHs, since none of them were affected by any change in concentration either in naphthalene or acenaphthene.

Appendix

Table 3.1. Ecoplate results for the different dilutions of rhizosphere soil of *Elusine coracana* plant growing in the uncontaminated soil.

Dilution												
10^{-2}					10^{-4}				10^{-6}			
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	+	+	+	+	+	-
B	+	+	+	+	+	+	+	+	+	-	+	+
C	+	+	+	+	+	+	+	+	+	+	-	-
D	+	+	+	+	+	+	+	+	-	+	+	+
E	+	+	+	+	+	_	+	+	-	+	+	-
F	+	+	+	+	+	+	+	_	+	-	-	-
G	+	+	+	+	+	+	-	-	-	+	+	-
H	+	+	+	+	+	+	+	+-	+	+	+	+

Table 3.2. Ecoplate results for the different dilutions of rhizosphere soil of *Biddens pilosa* plant growing in the uncontaminated soil.

Dilution

10^{-2}					10^{-4}				10^{-6}			
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	+	+	+	+	+	+
B	+	+	+	+	+	-	+	+	+	-	+	+
C	+	+	+	+	-	+	+	+	+	+	+	+
D	+	+	+	+	+	-	-	+	+	-	-	-
E	+	+	+	+	-	+	+	+	-	+	-	-
F	+	+	+	+	+	-	+	+	-	-	+	-
G	+	+	+	+	-	-	-	+	-	+	+	+
H	+	+	+	+	+	-	+	+	-	-	+	+

Table 3.3. Ecoplate results for the different dilutions of rhizosphere soil of *Elusine* coracana plant growing in the hydrocarbon contaminated soil.

Dilution												
10^{-2}					10^{-4}				10^{-6}			
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	+	+	+	+	+	-	-	+	-
B	+	+	+	+	+	+	+	+	-	_	+	+
C	+	+	-	+	+	+	+	+	+	+	-	+
D	+	+	+	+	+	+	+	+	+	-	+	+
E	+	+	+	+	+	+	+	+	+	+	-	+
F	+	-	+	+	-	+	-	+	+	-	+	+
G	+	+	+	+	+-	-	-	+	-	+	+	+
H	+	+	-	+	+	-	+	-	-	-	-	+

Table 3.4. Ecoplate results for the different dilutions of rhizosphere soil of *Biddens pilosa* plant growing in the hydrocarbon contaminated soil.

Dilution												
10^{-2}					10^{-4}				10^{-6}			
	1	2	3	4	5	6	7	8	9	10	11	12
A	-	+	+	+	-	-	+	+	-	+	-	-
B	+	+	+	+	+	+	+	+	+	-	+	-
C	+	+	-	+	+	-	+	+	+	+	-	-
D	+	-	+	+	-	+	-	+	+	+	-	+
E	-	+	+	+	+	+	+	+	+	+	-	-
F	+	-	+	+	+	-	+	+	+	-	-	-
G	+	+	-	+	-	+	-	-	+	-	+	-
H	-	-	-	-	-	-	-	-	+	+	+	+

Table 3.4. Substrates utilization for the different dilutions of rhizosphere soil of *E. coracana* and *B. pilosa* plants growing in contaminated and uncontaminated soils.

Dilutions	No. Substrates utilized	Plant species	Soil type
10^{-2}	29	*Elusine coracana*	Contaminated
10^{-4}	22		
10^{-6}	18		
10^{-2}	22	*Biddens pilosa*	Contaminated
10^{-4}	19		
10^{-6}	17		
10^{-2}	31	*Elusine coracana*	Uncontaminated
10^{-4}	25		
10^{-6}	20		
10^{-2}	31	*Biddens pilosa*	Uncontaminated

10^{-4}	22		
10^{-6}	19		